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## CORRECTION.

On page 581 of the last number of *The Journal of General Physiology*, Vol. V, No. 5, the statement was made that "The action of light was ascribed by Loeb to a chemical effect on the retinæ or some sensitive spots of the skin." There should be added the reference, *Arch. ges. Physiol.*, 1897, lxvi, 439.

# EXOSMOSIS IN RELATION TO INJURY AND PERMEABILITY.*

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, May 9, 1923.)

Since death involves exosmosis of substances from the cell it seems probable that if exosmosis can be accurately measured we may be able to follow the progress of death with sufficient exactness to afford a satisfactory basis of comparison with the results obtained from measurements of electrical conductivity. At the same time we may hope to gain direct evidence regarding the changes in permeability which accompany death.

The measurement of exosmosis by analyzing the solution which surrounds the cell involves obvious difficulties. These may be avoided by determining the concentration of dissolved substances in the cell. This can be done in a satisfactory manner in the case of *Nitella*,[1] whose sap can be obtained without contamination.

Since the determination of chlorides in the sap by the method described by Irwin[2] proved to be convenient and sufficiently accurate, it was employed for the measurement of exosmosis in these experiments.[3] The same amount of sap was used for each determination.

*The writer desires to express his thanks to the Carnegie Institution of Washington for a grant to cover the expenses of this investigation.

[1] The species employed was collected at Woods Hole, and pending identification is designated as "*Nitella* A." For the method of extracting the sap see Osterhout, W. J. V., *J. Gen. Physiol.*, 1921–22, iv, 275.

[2] Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 427.

[3] The temperature varied between 21° and 22°C. The temperature coefficient (between 2° and 21°C.) of exosmosis from dead cells (killed in pure chloroform and still retaining their chlorides) is not far from 1.3, as is to be expected. The temperature coefficient (between 13° and 18°C.) of exosmosis of chlorides from living cells placed in 0.3 per cent chloroform is about 1.8, which indicates that chemical processes are at work.

This was measured by drawing it up to a definite point in a standard capillary tube. This amount of sap expressed from normal cells[4] titrated against 30 drops[5] of the dilute $AgNO_3$ employed.

After exposure to an aqueous solution of chloroform,[6] it was found that some of the chlorides had diffused out and less $AgNO_3$ was required for titration. By plotting the number of drops required against the time of exposure we obtain a curve of exosmosis, as shown in Fig. 1.

It is evident that as long as the cell maintains its normal condition it retains all its chlorides, but that as soon as sufficient injury occurs the permeability increases and the chlorides begin to diffuse out.[7] The question arises whether we can measure this increase of permeability. The matter may be simplified by considering first of all what would happen if the cell should be killed and thus become completely permeable during the first instant of exposure to the solution of chloroform. In that case we might perhaps expect the process to follow the formula $K = \frac{1}{T} \log \frac{a}{a - x}$ (in which $a$ = the total amount of chlorides and $x$ = the amount which diffuses out in the time $T$) since this frequently happens with experiments on diffusion. The velocity constant, $K$, would then be expected to remain the same throughout; but when we follow the course of exosmosis in 0.4 per cent chloroform and calculate the velocity constant, we find that it steadily increases. If we assume that the outward diffusion of chlorides

[4] Cells 3 inches or more in length were employed. Only freshly collected material, of normal appearance, was used. The criteria of normality are described in a previous paper (*cf.* Foot-note 1).

[5] The actual concentration of the chlorides in the sap was about 0.128 M (*cf.* Foot-note 2).

[6] The chloroform was dissolved in distilled water of pH 6.2; at the concentrations employed the chloroform produced very little change in this pH value. From twenty to thirty cells were placed in about 1,500 cc. of solution; this was stirred during exposure. When the exposure was finished the cells were removed and very quickly freed from adhering solution by means of cloth or filter paper; the sap was then extracted from each cell as described in a previous paper[1] and titrated.

All concentrations of chloroform are stated as percentages by volume.

[7] *Cf.* Foot-note 2; also Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

follows this equation whenever the permeability remains constant during the whole process, we should interpret any increase of the velocity constant to indicate an increase of permeability, and the velocity constant would be regarded as a measure of permeability.

It is a simple matter to determine the velocity constant at any time on the supposition that we have to do with a process which

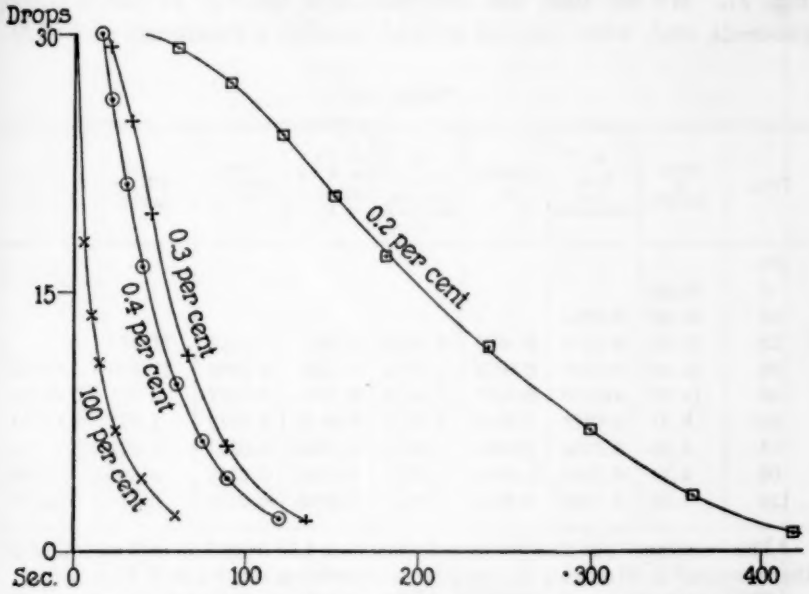


FIG. 1. Curves of exosmosis of chlorides in aqueous solutions of chloroform (with the exception of the curve for 100 per cent, in which case the cells were killed in pure chloroform and then placed in distilled water to permit exosmosis to take place); the ordinates show the concentration of chlorides in the cell sap (expressed as number of drops of $AgNO_3$ required for titration).

Each point represents the average of twenty or more experiments; the probable error of the mean is less than 1.5 per cent of the mean.

behaves as a monomolecular reaction whose velocity constant is increasing (as would be the case if the temperature were rising). For example, we see that after an exposure of 22 seconds to 0.4 per cent chloroform, 26.25 drops[8] of $AgNO_3$ are required; measuring

[8] The fact that the results are expressed in fractional parts of a drop is due to the averaging of several determinations.

back on the curve we find that at 20 seconds 27.1 drops were necessary. To find the velocity constant we may proceed as if the reaction started at 20 seconds. Employing the formula given above, and putting $a = 27.1$ and $a - x = 26.25$, we obtain (using common logarithms) 0.007 as the velocity constant. Proceeding in this manner we obtain the values given as $K_s$ in Column 5 of Table I (see also Fig. 2). We see that the constant rises steadily as the reaction proceeds, and, when the cell is dead, reaches a maximum at 0.0129.

TABLE I.

| Time. | Drops of $AgNO_3$ | $K_c$ * from pure chloroform. | $0.0596 - K_c$ | $K_s$ † from 0.4 per cent chloroform. | $K_p = K_s + 0.0596 - K_c$ | "Constants"‡ for $K_p$ | § $1 \div K_p - 16.779$ | "Constants"‖ for $1 \div K_p$ |
|---|---|---|---|---|---|---|---|---|
| *sec.* | | | | | | | | |
| 0 | 30.00 | | | | | | | |
| 15 | 30.00 | 0.0596 | 0 | | | | | |
| 22 | 26.25 | 0.0519 | 0.0077 | 0.0070 | 0.0147 | 0.0176 | 51.250 | |
| 30 | 21.30 | 0.0418 | 0.0178 | 0.0110 | 0.0288 | 0.0190 | 17.940 | 0.05697 |
| 40 | 16.50 | 0.0319 | 0.0277 | 0.0115 | 0.0392 | 0.0187 | 8.731 | 0.04271 |
| 60 | 9.53 | 0.0179 | 0.0417 | 0.0125 | 0.0542 | 0.0232 | 1.671 | 0.03913 |
| 75 | 6.30 | 0.0153 | 0.0443 | 0.0125 | 0.0568 | 0.0222 | 0.827 | 0.03382 |
| 90 | 4.14 | 0.0143 | 0.0453 | 0.0129 | 0.0582 | 0.0218 | 0.403 | 0.03094 |
| 120 | 1.88 | 0.0135 | 0.0461 | 0.0129 | 0.0590 | 0.0189 | 0.170 | 0.02530 |

* This "constant" was obtained by reckoning back 1.25 seconds in each case; thus for the "constant" at 30 seconds the reaction was regarded as starting at 28.75 seconds.

† This "constant" was obtained by reckoning back 2 seconds in each case; thus for the "constant" at 30 seconds the reaction was regarded as starting at 28 seconds.

‡ In calculating we subtract 15 seconds from the time given in Column 1 and put $x = K_p$ and $a = 0.0596$.

§ We subtract 16.779 ( $= 1 \div 0.596$) because this is the base line to which the curve falls.

‖ In calculating we subtract 22 seconds from the time given in Column 1 and put $a = 51.25$ and $1 \div K_p = a - x$.

We might infer from this that when the cells are dead and completely permeable, the velocity constant at 22°C. will in all cases be 0.0129. In order to test this it is desirable to study the exosmosis of cells which have been made completely permeable by killing, but which have not yet lost their chlorides. For this purpose the surfaces of living cells were carefully freed from adhering drops of water

## CORRECTION.

The accompanying charts are to replace Figs. 2 and 3 on pages 713 and 717, respectively, in Vol. v, No. 6, July, 1923. The charts are arranged to be pasted over the place occupied by the figures in the text at present. The figure number is given on the back of each chart.

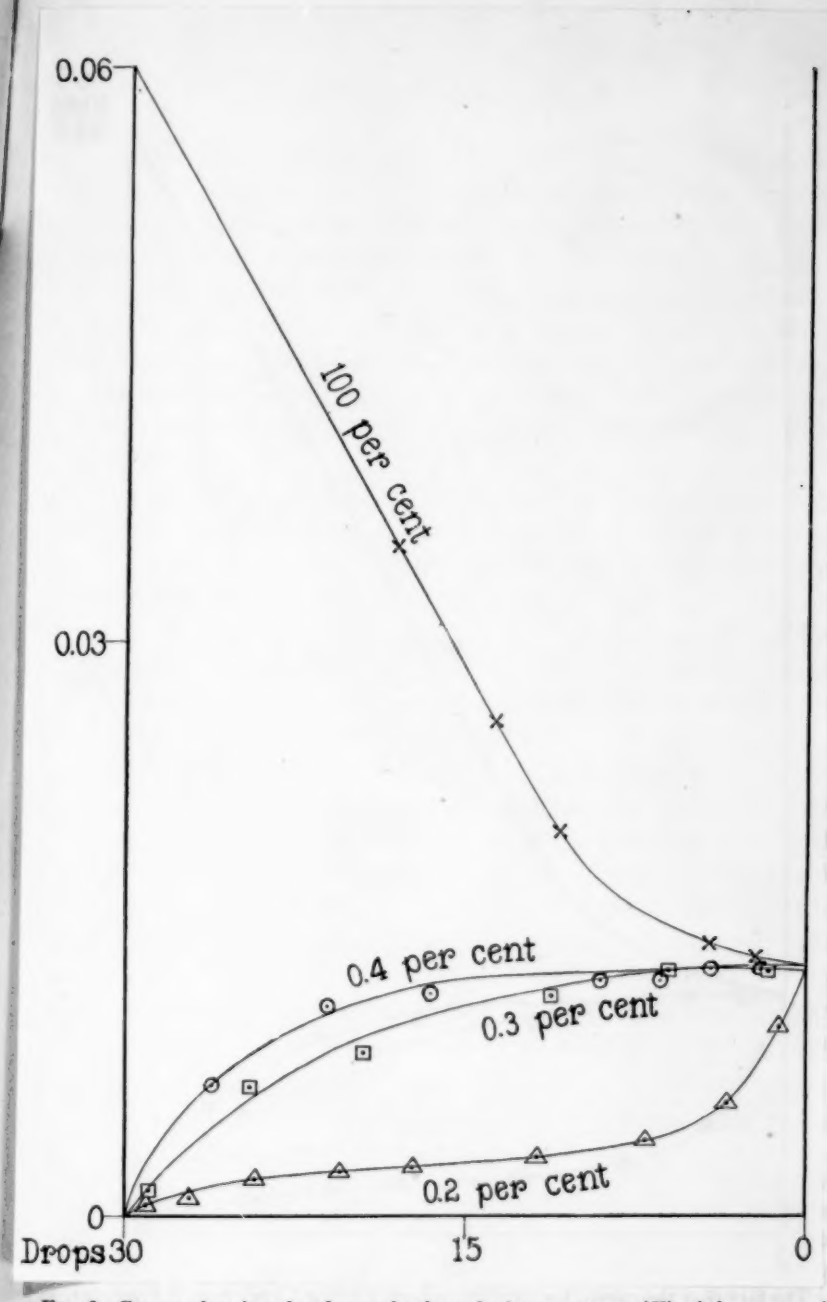


FIG. 2. Curves showing the change in the velocity constants ($K$) of the curve of exosmosis shown in Fig. 1. The velocity constants are plotted as ordinates; they were calculated by means of the formula $K = \frac{1}{T} \log \frac{a}{a-x}$. The abscissæ represent the stage of the reaction as denoted by the concentration of chlorides in the sap (expressed as number of drops of $AgNO_3$ required for titration).

by means of filter paper and placed for 10 minutes in pure chloroform. This killed the cells without permitting exosmosis of chlorides; when such cells were removed from chloroform the sap required 30 drops of $AgNO_3$ for titration, which showed that the cells contained chlorides in normal amount.

When such cells (freed from adhering drops of chloroform) are placed in distilled water (at pH 6.2) the chlorides diffuse out and the constants of the curve of exosmosis may be calculated as described above. We find that these constants diminish during the progress of exosmosis as shown in Column 3 ($Kc$) of Table I and in the upper curve in Fig. 2. As this curve is a straight line in the upper part of its course we may extrapolate it to the start of the reaction (at 30 drops); we thus obtain the value 0.0596, which may be regarded as the velocity constant when the cell has become completely permeable but has not yet lost its normal content of chlorides. But as soon as the chlorides begin to diffuse out it is evident that something happens which diminishes the velocity constant.[9]

Inasmuch as we have already found that during the death of the cell the velocity constant increases, we may, for the purpose of analyzing the mechanism concerned, proceed as if during the course of death two processes occur; (1) an increase in the velocity constant due to an increase in permeability, and (2) a decrease in the velocity constant which may be regarded as quite independent, since it occurs after the cell is dead and the plasma membrane is destroyed. Hence if we wish to follow the changes in permeability we must ascertain what the velocity constant would be if the second process were lacking. This may be done by adding to the apparent velocity constant

[9] This is apparently due to the shape of the cell since the velocity constant also falls off when NaCl diffuses out of a cylindrical thimble of parchment paper having approximately the shape of the cell. In this case the graph was curved from the start so that it is quite possible that in extrapolating the curve for pure chloroform we should have taken a value somewhat higher than 0.0596, but this probably would have little effect on the form of the time curve for permeability since it would probably involve only a small and constant addition to each ordinate.

The fact that the curves for pure chloroform and for 0.4 per cent, 0.3 per cent, and 0.2 per cent chloroform in Fig. 2 approach the same value at the end is due to the fact that they are approaching the same condition in respect to permeability and content of chlorides.

in 0.4 per cent chloroform the amount which has been subtracted from it by the operation of the second process. This becomes a simple matter if we plot the velocity constants against the number of drops as in Fig. 2. In this way we can determine at any stage of the reaction, *e.g.* when half completed (at 15 drops), both the apparent velocity constant and the loss due to the second process. By adding these together we obtain the value which the velocity constant would have if the second process were lacking. This value may be assumed to express the permeability of the cell. It is small at the beginning[10] and rises during the progress of death (as shown in Table I, Column 6 ($K_p$) and in Fig. 3),[11] indicating an increase in permeability, such as would be expected when the cell is injured.

If this curve actually represents the increase in permeability which occurs during death it is evident that it would be obtained directly if the diffusion process always followed the equation $K = \frac{1}{T} \log \frac{a}{a-x}$ whenever the permeability remained constant during the entire process of diffusion; we find, however, that when the permeability remains constant[12] the outward diffusion of chlorides does not follow this equation but behaves in such a manner that the value which expresses the permeability is lowered by a definite amount at each stage of the process. We must therefore correct for this by adding to the value which expresses the permeability the precise amount which this lowering has subtracted from it. This may be done in the manner described above.

As an example of the method of calculation we may take the velocity constant at 22 seconds in 0.4 per cent chloroform. The number of drops is 26.25 and the velocity constant is 0.007. At the corresponding stage of the process in pure chloroform (*i.e.* at 26.25 drops) the

[10] The fact that the cell is not wholly impermeable to chlorides is indicated by the results of Hoagland and Davis (Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629), and by the fact that cells placed in a solution of chlorides conduct an electric current as described later in this paper.

[11] In order to facilitate comparison, the ordinates in Fig. 3 are multiplied by 1.678, and 15 seconds are subtracted from the time values; this subtraction merely causes the curve to rise at once instead of after a latent period.

[12] *I.e.*, when the cell has been killed without losing its chlorides.

velocity constant has been lowered from 0.0596 (its value at the start of the reaction) to 0.0519. The amount of this lowering (0.0596 — 0.0519 = 0.0077) must be added to 0.007 giving 0.0147. Proceeding in this manner we obtain the series of velocity constants[13] designated as $K_p$ in Table I; these give, when plotted against time, the curve shown in Fig. 3.

[13] It would, of course, be possible to make the calculation in a different manner. We find, for example, that a similar curve is obtained when the velocity constant $K_p$ is calculated from the equation for a bimolecular reaction. In this case the constants rise both in 0.4 per cent chloroform and in pure chloroform from the start. Let us first consider the constants for the curve for the pure chloroform. The value of the constant at the start (obtained by extrapolation) is 0.0028. Any rise above this as there reaction proceeds is independent of changes in permeability (since it occurs in a dead cell which is completely permeable), and hence must be subtracted from the apparent rise of permeability which occurs in a living cell placed in 0.4 per cent chloroform. When this is done we get a curve of approximately the same form as that already obtained, but which is less regular and which does not agree so well with the results obtained by the measurement of electrical conductivity, as explained later. The curve already obtained (by calculating the constants from the monomolecular formula) therefore seems preferable.

As an example of the method of calculation we may take the curve for 0.4 per cent chloroform; after an exposure of 22 seconds (as already mentioned) 26.25 drops were required and, measuring back, we find that at 20 seconds 27.1 drops were needed. We may regard the reaction as starting at 20 seconds, and proceed to find the constant. If we suppose that in the bimolecular reaction $a + b = c$ the combining substances, $a$ and $b$, are of the same molecular concentration (*i.e.* $a = b$) we may use the formula

$$K = \frac{1}{at}\left(\frac{(x)}{(a - x)}\right)$$

in which $t$ = time and $x$ = the amount of $c$ produced. If at 20 seconds the values of $a$ and $x$ are $a_{20}$ and $x_{20}$, while at 22 seconds they are $a_{22}$ and $x_{22}$ we may write

$$K = \left(\frac{1}{22 - 20}\right)\left(\frac{x_{22} - x_{20}}{a_{20} - a_{22}}\right)$$

$$K = \left(\frac{1}{22 - 20}\right)\left(\frac{1}{a_{22}} - \frac{1}{a_{20}}\right)$$

$$K = \frac{1}{2}\,(0.038095 - 0.0369) = 0.000598$$

Proceeding in this manner we obtain a series of values which may be compared with the curve obtained by calculation on a monomolecular basis.

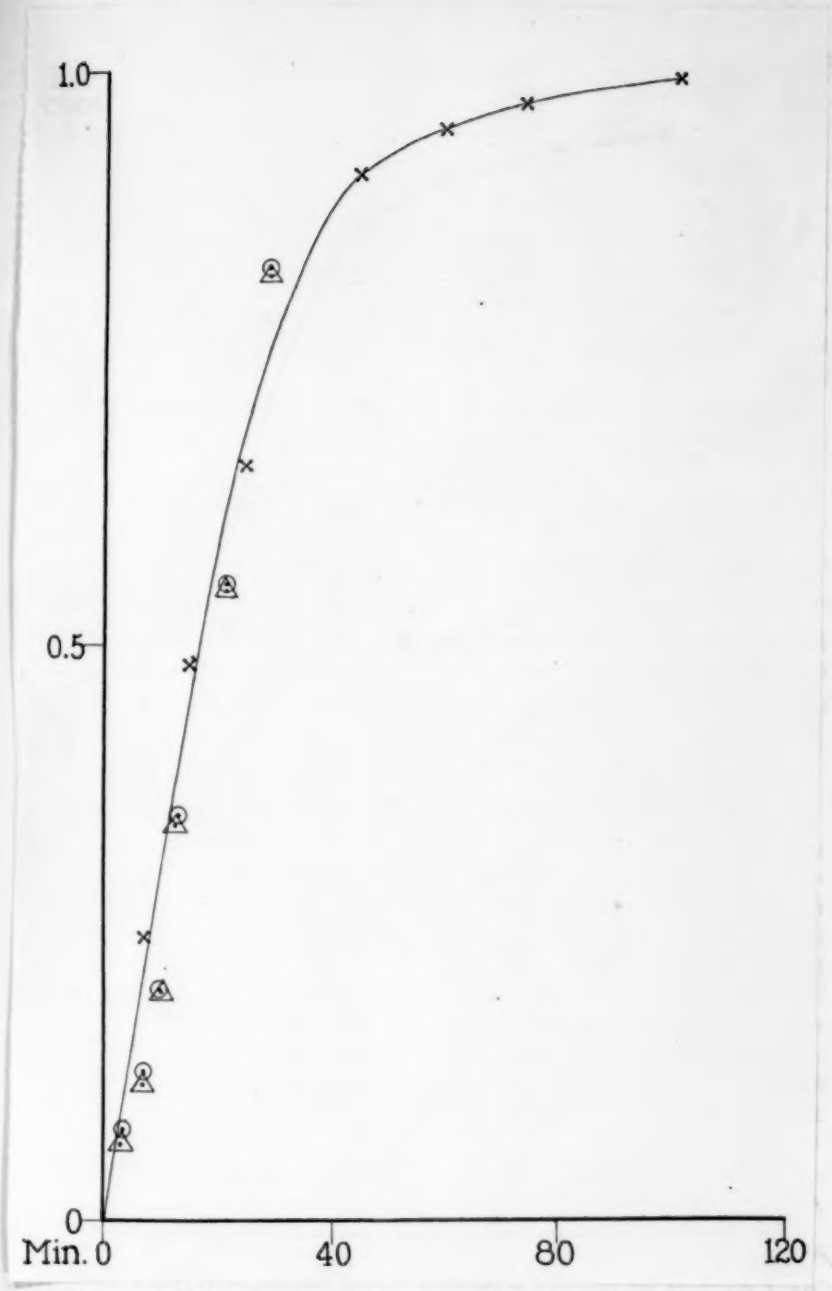


FIG. 3. Curve showing the increase in permeability during the progress of death. The crosses are derived from the measurements of exosmosis; the circles and triangles from determinations of electrical conductivity. The ordinates express permeability; complete permeability (as found in dead cells) is taken as unity.

It is desirable that this curve be compared with that obtained from measurements of electrical conductivity, which likewise shows the increase of permeability which takes place during the process of death. For this purpose determinations of net electrical resistance were made as described in a previous paper.[14] The cells (from 50 to 100 in each case) were closely packed and the current was sent through at right angles to the long axes[15] of the cells. The results[16] are shown in the curve in Fig. 4.

It has been shown that similar curves which are obtained in the case of *Laminaria*[17] may be expressed by the formula

$$\text{Resistance} = 2{,}700\left(\frac{K}{K_2 K_1}\right)\left(e^{-K_1 T} - e^{-K_2 T}\right) + 90\, e^{-K_2 T} + 5$$

This formula also applies in the present case, as is evident from Fig. 4, where the curve shows the values obtained by this formula and the points show the observed values. In this case[18] we put $K_1 = 0.1$ and $K_2 = 3.15$. In the formula, 5 is added because this is the net resistance of the dead tissue,[19] and therefore represents the base line to which the curve of resistance falls.

[14] *Cf.* Foot-note 1.

[15] When the current is sent through lengthwise the results are irregular. This may be due to the fact that as the cells die they lose their stiffness, and the ends, which are not supported, droop and collapse. Before the determinations were made the cells were placed for some time in fifteen parts sea water plus 85 parts distilled water (or in 25 parts sea water plus 75 parts distilled water, which had approximately the conductivity of the cell sap), and the measurements were made in this solution. If the addition of chloroform produced a change in the conductivity this was corrected by the addition of sea water.

[16] The following values were obtained (expressing all resistances as per cent of the normal). At 5 minutes, 61.5 per cent; 10 minutes, 39 per cent; 15 minutes, 24 per cent; 20 minutes, 14 per cent; 30 minutes, 9 per cent, 40 minutes, 6 per cent. All measurements were made at 21°C., or corrected to this figure.

[17] *Cf.* Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Philadelphia and London, 1922, 61.

[18] If the constant of the curve is calculated by the monomolecular formula it will be found to fall from the start. This is the case when $K_2 \div K_1 > 31$. When $K_2 \div K_1 = 31$ the constant is the same throughout the curve; when $K_2 \div K_1 < 31$ the constant rises from the start. *Cf.* Foot-note 17, p. 78.

[19] All resistances are expressed (as in the case of *Laminaria*) as per cent of the normal.

It is evident that as the resistance of the protoplasm to the passage of ions decreases, its permeability to ions must increase, and *vice versa*. We may therefore regard the reciprocal or resistance (conductance) as a measure of permeability.[20] If we take the reciprocals

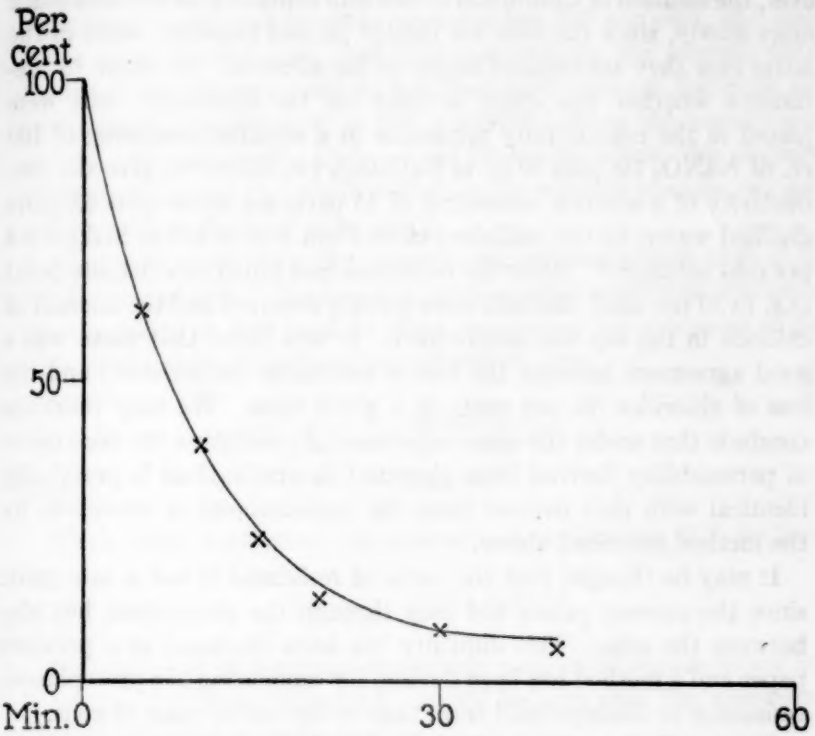


FIG. 4. Curve showing the electrical resistance of *Nitella* in 0.4 per cent chloroform (expressed as per cent of the normal). Each point represents the average of ten or more experiments; the probable error of the mean is less than 10 per cent of the mean.

of the values shown in Fig. 4, and plot them beside[21] the values for $K_p$ we obtain the points shown as circles in Fig. 3. It is evident that there is a very good agreement between this curve, which expresses

[20] *Cf.* Foot-note 17, Chapter VI.

[21] In order to facilitate comparison, the ordinates have been multiplied by 5 and the abscissæ by 0.0121.

the permeability as measured electrically, and the curve of $K_p$ which expresses permeability as determined by the measurement of exosmosis. The chief difference is that in the former the change of permeability is much slower than in the latter. In the former case, however, the solution of chloroform comes into contact with the cells much more slowly, since the cells are tightly packed together, while in the latter case they are exposed singly to the solution. In order to determine whether this could account for the difference, cells were placed in the conductivity apparatus in a solution consisting of 100 cc. of $NaNO_3$ 1M, plus 30 cc. of $Ca(NO_3)_2$ 1M, diluted to give the conductivity of a solution consisting of 15 parts sea water plus 85 parts distilled water; to this sufficient chloroform was added to make a 0.4 per cent solution.[22] After the resistance had fallen to a definite point (*e.g.* to 50 per cent) the cells were quickly removed and the amount of chloride in the sap was determined. It was found that there was a good agreement between the loss of resistance (in per cent) and the loss of chlorides (in per cent) in a given time. We may therefore conclude that under the same experimental conditions the time curve of permeability derived from electrical determinations is practically identical with that derived from the measurement of exosmosis by the method described above.

It may be thought that the curve of resistance is not a safe guide since the current passes not only through the protoplasm but also between the cells. This difficulty has been discussed in a previous paper and a method has been devised for calculating the protoplasmic resistance as distinguished from that of the whole mass of cells.

If we call the current which passes through the protoplasm $C_P$, and that which passes between the protoplasmic masses (through the cell wall and the spaces between the cells) $C_W$, we may write

$$\text{Total current} = C = C_P + C_W$$

or, in terms of resistance,

$$\text{Total current} = C = \frac{1}{R} = \frac{1}{R_P} + \frac{1}{R_W}$$

[22] In this mixture exosmosis was as rapid as in 0.4 per cent chloroform in distilled water. When the chloroform was omitted the cells lived well in the mixture.

where $R$ is the total net resistance of the mass of cells, $R_P$ the resistance of the protoplasm, and $R_W$ the resistance of the cell wall and the spaces between the cells.

We find that when the cells are placed in solutions[23] of $La(NO_3)_3$, HCl, or alum,[24] the resistance rises above the normal, (which is taken as 100 per cent) and in some cases reaches 350 per cent, and since some of the current must flow in the protoplasm, the amount which traverses the cell wall must be less than this. We are therefore safe in putting $C_W$ as low as $1 \div 400 = 0.0025$. Since the conductance of the cell wall suffers little or no alteration where the chemical character (but not the conductivity) of the solution changes, we may take 0.0025 as the fixed value of $C_W$.

We can now calculate the protoplasmic resistance from that of the whole mass of cells. For example, under normal conditions the resistance is taken as 100 (and all other resistances are expressed as per cent of the normal) and we may therefore write

$$C = \frac{1}{100} = 0.0025 + C_P$$

Whence

$$C_P = 0.0075$$

When, after 5 minutes, the resistance has fallen to 61.5 we have

$$C = \frac{1}{61.5} - 0.0025 = 0.0138$$

Proceeding in this manner we obtain a series of values which are plotted in Fig. 3 as triangles.[25] It will be seen that the agreement with the curve of $K_p$ is fairly close.

It is therefore evident that the form of the time curve of permeability is practically the same whether it is derived from measurements of exosmosis, or of protoplasmic resistance, or of the resistance of the whole mass of cells. This fact would seem to indicate that it is not far from a true picture of the process actually taking place. It may

[23] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1921–22, iv, 1; also Foot-note 17.

[24] All of these solutions had the same conductivity as the dilute sea water in which the normal resistance of the cells was measured.

[25] In this case the abscissæ are multiplied by 0.0121, while the ordinates are multiplied by 5.0633.

be added that the general character of the curve is what we might expect on the basis of evidence from derived plasmolysis, penetration of dyes, and other methods.

We may calculate the constants for the time curve of permeability, (using the values of $K_p$ from Table I) according to the monomolecular formula, putting $a = 0.0596$ and $a - x = 0.0596 - K_p$, and subtracting 15 seconds to offset the latent period. We thus obtain the constants given in Column 7 of Table I. It is evident, therefore, that the time curve of permeability agrees with a reaction of the first order, somewhat inhibited at the start. If the latent period were not subtracted the constants would be still smaller at the start and the agreement with the curves obtained from electrical measurements would be still closer.

The curves of permeability for 0.3 per cent and 0.2 per cent chloroform are quite similar in form to that for 0.4 per cent, notwithstanding the fact that the curves for the constants of their curves of exosmosis differ (as shown in Fig. 2).

It would, of course, be possible to compare the curve of electrical resistance with the similar curve obtained from measurements of exosmosis by taking the reciprocal of $K_p$ (see Column 8, Table I). The difficulty arises that we do not know the value of this reciprocal at the start. For this reason comparison is unsatisfactory. If we calculate the constant of the curve of $1 \div K_p$ from the formula $K = \frac{1}{T} \log \frac{a}{a - x}$ we find that it falls as the reaction proceeds (Column 9, Table I); this is also the case with the curve of electrical resistance, and as the latter follows the formula involving consecutive reactions, it appears highly probable that this is also the case with the curve of $1 \div K_p$.

We may now consider a feature of exosmosis, which has not hitherto been discussed, namely the latent period at the start. In the case of 0.4 per cent chloroform there is a latent period of 15 seconds (Fig. 1) during which there seems to be no reaction; in the case of 0.3 per cent it is longer,[26] and in that of 0.2 per cent it is longer still. It might

[26] The curve for 0.3 per cent was made with younger material than that employed in the case of the other curves. With older material exosmosis would probably have been slower and the latent period longer. The actual values of time in the

be suggested that this is connected with the decrease in permeability (as determined by electrical conductivity) which has been observed in some organisms immediately after the application of chloroform.[17] This, however, does not seem to occur in *Nitella* (as shown by electrical measurements). We find, moreover, that there is also a latent period when the cell is killed by reagents such as $NH_4OH$ and NaOH, which produce no decrease in permeability (as determined by electrical measurements). The fact that the latent period is less with $NH_4OH$ than with NaOH may be correlated with the more rapid penetration of the former. It is evident that the latent period might be due to a variety of causes and that further experiments will be necessary to determine the real one.[27]

The question arises whether this method of studying exosmosis can be applied in the case of cells whose sap cannot be obtained without contamination. In such cases we must resort to tests of the external solution. It therefore seems desirable to measure exosmosis in *Nitella* by testing the external solution and to compare the results with those obtained by analysis of the sap.

For this purpose cells were placed[28] in about ten times their volume of 0.4 per cent chloroform, and allowed to remain for 2 minutes.[29] They were then removed[30] to another dish containing the same amount of solution; after 2 minutes they were removed to a new dish of solution, and so on until 10 minutes had elapsed. After this they were transferred every 5 minutes until 20 minutes more had elapsed and thereafter the transfers were made at lengthening intervals. In this

experiment were divided by 1.33 to correct for temperature. These corrected values are shown in Fig. 1, and the constants from the corrected curve are shown in Fig. 2.

[27] In the experiments on the electrical conductivity of *Nitella* a latent period was not observed (except as an occasional irregularity). This may possibly be due to the fact that the time relations of the whole process of death are altered by the close packing of the cells in the conductivity apparatus.

[28] These experiments were made with another species, designated temporarily as "*Nitella* C."

[29] The solution was stirred during the exposure.

[30] In some cases the cells were dipped for an instant into a fresh solution of chloroform at each transfer in order to remove any chlorides present in the adhering drops.

way chlorides were prevented from accumulating sufficiently in the external solution to influence the results, and the experimental conditions were therefore similar to those described in the beginning of this paper, where a large volume of solution was employed in order to prevent the concentration of chlorides from increasing sufficiently to cause an error.

The chlorides in the external solution were determined by titration in the manner previously described. When an interval of 20 minutes did not produce enough chlorides to require more than 1 drop of $AgNO_3$ the process was regarded as completed.[31]

The results, while showing a general agreement with those obtained by titrating the expressed sap, were much more irregular. It is not improbable, however, that this may be obviated by improved technique.[32]

It is quite possible that in applying this method to other organisms, curves of exosmosis may be obtained which are decidedly different from those found in the case of *Nitella*. One possible reason for such differences may lie in the fact that in *Nitella* the chlorides are mostly combined with inorganic cations[33] which can readily diffuse out when the cell is injured. If, however, they were partly combined with substances which diffuse out slowly or not at all (except after chemical change, as in the case of some proteins) the curve of exosmosis would be different from that found in *Nitella*.

The method of testing the external solution has been employed by several authors,[34] the most recent paper which has come to the

[31] In case we fail to regard the process as complete when it is really finished we shall overestimate the total amount of chloride which diffuses out, and this will make the constants of the curve of exosmosis appear to diminish toward the end.

If some of the chlorides were in combination with substances which diffuse out readily, while the rest were combined with substances which diffuse out slowly, it would be more difficult to determine the total amount of chlorides by testing the external solution than by testing the expressed sap, since in the latter case all the chlorides would be measured.

[32] In these experiments whole plants were used, including cells of all sizes. If selected cells of uniform size were employed the results might be more regular.

[33] *Cf.* Foot-note 1; also Hoagland, D. R., and Davis, A .R., *J. Gen. Physiol.*, 1922–23, v, 629.

[34] Brooks, S. C., *Am. J. Bot.*, 1916, iii, 483. Stiles, W., and Jörgensen, I., *Ann. Bot.*, 1915, xxix, 349. Medes, G., and McClendon, J. F., *J. Biol. Chem.*, 1920, xlii, 541.

writer's attention, being that of Gray,[35] who finds that the process of exosmosis is at first slow, then more rapid, and finally slows down. This was also the case in the experiments of Stiles and Jörgensen,[36] and in those of the writer.

We may summarize these results by saying that whether we study the process of death by measuring exosmosis or by determining the electrical conductivity of the cell, we arrive at the same result. It has been shown in previous papers[17] that the death curve may be expressed as the result of a series of consecutive reactions. This leads to a quantitative theory of the death process. As a result of this theory we have come to regard the death process as one which is always going on under normal conditions but which produces no disturbance of life processes unless it is unduly accelerated by an injurious agent. If this interpretation is correct a study of death may be expected to throw considerable light on the normal life processes. From this standpoint the study of death becomes very important, and it is therefore to be hoped that the method outlined above, of employing measurements of exosmosis for this purpose, may prove to be generally applicable.

SUMMARY.

The time curve expressing the increase in the permeability of *Nitella* during the progress of death is practically the same whether derived from measurements of exosmosis or of electrical resistance.

[35] Gray, J., *J. Physiol.*, 1921, lv, 322.

[36] Stiles, W., and Jörgensen, I., *Ann. Bot.*, 1917, xxxi, 47.

# THE PENETRATION OF DYES AS INFLUENCED BY HYDROGEN ION CONCENTRATION.

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The purpose of this investigation is to analyze the mechanism by which dyes are taken up by the cell under the influence of different concentrations of hydrogen ion.

## I.

## *Methods.*

The technique has been described in a previous paper.[1] The plant employed was *Nitella*, whose very long, multinucleate cells contain a large, central vacuole filled with sap which is surrounded by a delicate layer of protoplasm. If the end of the cell is cut off, and gentle pressure applied, the clear sap flows out, free from protoplasm or chlorophyll granules. If the cell has been previously placed in a dye which has penetrated through the layer of protoplasm into the cell the concentration of the dye in the sap may be measured in a very simple manner. A drop of the sap is taken up into a capillary tube and the color is then compared with the colors of other tubes, of the same diameter, containing different concentrations of the same dye.

The dye used is brilliant cresyl blue (from the National Aniline and Chemical Company).[2] As it was not completely soluble in water the stock solution was made up by placing 2 gm. of dye in 2 liters of distilled water, dissolving as much as possible and filtering off the residue. Although the actual concentration was not known, this is

[1] Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 223.

[2] It is often impossible to compare measurements made with the same dye obtained from different manufacturers, since there is apt to be too much variation in the composition.

of no consequence, since in these experiments only relative concentrations were important. This stock solution was called 0.1 per cent and the other percentages mentioned in the paper are to be understood as representing corresponding dilutions of this stock solution.

All the experiments were made at 20 ± 0.1°C. Every determination made for the amount of dye present in the sap, is an average of forty experiments.

To make up buffer solutions, the standard buffer mixtures were diluted to $\frac{m}{100}$ (this did not appreciably change the pH value in any case). From pH 3 to pH 5 phthalates were employed; from pH 5.6 to pH 8, phosphates; from pH 8 to pH 9, borates. Check experiments showed that at the same pH value phthalates had the same effect as phosphates, and phosphates the same as borates, so that their effect on the cells must be due to hydrogen ion concentration, and other differences in chemical composition have little influence.

## II.

### *Absorption of Dye by the Cellulose Wall.*

In studying the penetration of a dye into the vacuole of *Nitella*, both the cellulose wall and the protoplasmic layer must be considered, since the dye must diffuse through these two layers before it reaches the vacuole.

The protoplasmic layer is so very thin that it is not possible to determine accurately to what extent it stains with the dye.

At first sight it might seem that the rate of penetration of the dye into the vacuole might depend on the amount of absorption of the dye by the cell wall so that the more the dye is taken up by the cell wall, the less it enters the vacuole. It was necessary, therefore, to carry out experiments to determine whether this is the case.

The results of some of these experiments are shown in Table I. It is evident that at low pH values (pH 5.6) the cellulose wall takes up the stain rapidly, while the sap acquires it very slowly. Between pH 8 and 9 the cell wall stains very slightly, while the sap takes up the stain rapidly. It is therefore evident that if there is any competition for the stain between the sap and the cell wall it would be most effective at the lower pH values. It is evident, however, from

Experiments 1 and 2 that at pH 5.6 the cell wall ceases after a few minutes to take up the stain and the competition must therefore be confined to the first few minutes of the experiment. As all of the experiments at lower pH values (pH 6.6 and 7) were long (lasting for several hours) this competition, if it really exists, is a negligible factor.

TABLE I.

*Relation between the Absorption of the Dye by Cellulose Wall and by Cell Sap.*

| Experiment No. | pH | Time of exposure. | Concentration of dye in outside solution. | Concentration of dye in vacuole. | Color of cellulose wall. |
|---|---|---|---|---|---|
| | | | *per cent* | *per cent* | |
| 1 | 5.6 | 40 min. | 0.004 | 0 | Sky-blue.* |
| 2 | 5.6 | 24 hrs. | 0.004 | 0.0005 | " * |
| 3 | 6.6 | 22 " | 0.002 | 0.0017 | Medium blue.* |
| 4 | 7.0 | 14 " | 0.002 | 0.007 | " " * |
| 5 | 7.7 | 12 " | 0.002 | 0.06 | Pale blue.* |
| 6 | 5.6 | 40 min. | 0.002 | 0 | " " |
| 7 | 8.0 | 50 " | 0.002 | 0.04 | No stain. |
| 8 | 9.0 | 50 " | 0.002 | 0.096 | " " |

* Signifies that the color of the cellulose wall was deeper than that of the outside solution.

III.

### *Penetration of Dye without Injury.*

To study the penetration of the dye into the vacuole the cells were placed in an aqueous solution of cresyl blue (0.002 per cent) at pH 6.6 and 7.

At these pH values, the dye was taken up until an equilibrium was reached (Fig. 1 and Table II) beyond which no accumulation of dye occurred. In Fig. 1, the observed values are represented by symbols, and the curves show the values calculated from the equation representing reaction of the first order, $K = \frac{1}{t} \log \frac{a}{a - x}$, in which $a$ = the amount of dye present in the cell sap at the end of the reaction when equilibrium is reached; $x$ = the amount of dye present

in the cell sap at the time $t$; $a - x$ = the additional amount of dye which must accumulate in the sap before equilibrium is reached, and $K$ = the velocity constant. For pH 6.6, $a$ = 0.0017 per cent, and $K$ = 0.00182, while for pH 7, $a$ = 0.007 per cent, and $K$ = 0.00186 (Table II). It is evident that the observed values agree with the calculated quite closely.

The fact that the process behaves as a reaction of the first order might suggest that it is due merely to diffusion since diffusion experi-

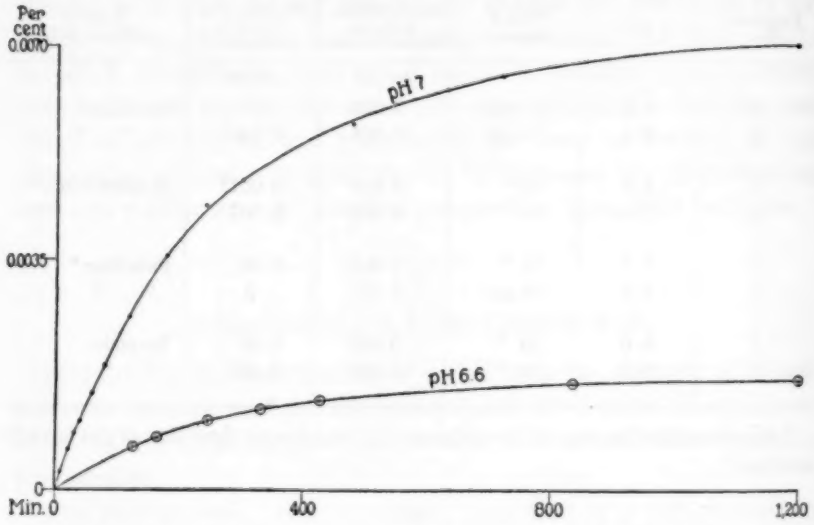


FIG. 1. Curves showing the penetration of 0.002 per cent cresyl blue solution into living cells of *Nitella* at pH 6.6 and pH 7. The concentrations of dye (in per cent) in the cell sap are plotted as the ordinates, and the time in minutes as the abscissæ. The smooth curves show the calculated values, while the symbols represent the observed values.

ments are commonly arranged in such a manner that the process behaves as a reaction of the first order. We find, however, that the temperature coefficient is high (in the neighborhood of 3),[3] which indicates that we are dealing with a chemical reaction which is slower than the process of diffusion and which therefore determines the temperature coefficient.

[3] Results on the temperature coefficient will appear in a later paper.

It is evident that the final equilibrium is higher at pH 7 than at pH 6.6. In order to explain this we may assume that the dye in the vacuole is combined with a protein[4] which acts somewhat like gelatin, as described by Loeb,[5] in that the increase of pH from 6.6 to 7.0 causes an increase in the amount of active or ionized protein which can combine with the dye (and a corresponding decrease of inactive

TABLE II.

*Penetration of 0.002 Per Cent Solution of Cresyl Blue into Living Cells of Nitella at 20°C. at pH 6.6 and pH 7.*

| pH 7 ($a$ = 0.007 per cent) | | | | pH 6.6 ($a$ = 0.0017 per cent) | | | |
|---|---|---|---|---|---|---|---|
| $t$ observed. | $X$ | $K=\frac{1}{t}\log\frac{a}{a-x}$ | $t$ calculated for $K$=0.00186 | $t$ observed. | $X$ | $K=\frac{1}{t}\log\frac{a}{a-x}$ | $t$ calculated for $K$ = 0.00182 |
| *min.* | *per cent* | | *min.* | *min.* | *per cent* | | *min.* |
| 10 | 0.0003 | 0.00190 | 10.3 | 120 | 0.0007 | 0.00193 | 126 |
| 20 | 0.00065 | 0.00211 | 22.8 | 180 | 0.00086 | 0.00170 | 167 |
| 30 | 0.0009 | 0.00198 | 32.2 | 240 | 0.0011 | 0.00188 | 247 |
| 40 | 0.0011 | 0.00185 | 40.1 | 360 | 0.00128 | 0.00168 | 332 |
| 60 | 0.00155 | 0.00181 | 58.5 | 480 | 0.00142 | 0.00163 | 428 |
| 80 | 0.002 | 0.00182 | 79.0 | 720 | 0.00165 | 0.00212 | 835 |
| 120 | 0.0028 | 0.00184 | 120.0 | 1,200 | 0.0017 | | |
| 180 | 0.0038 | 0.00188 | 178.0 | 2,000 | 0.0017 | | |
| 240 | 0.0046 | 0.00193 | 250.0 | Average..... | | 0.00182 | |
| 300 | 0.005 | 0.00182 | 294.0 | | | | |
| 480 | 0.0059 | 0.00167 | 435.0 | | | | |
| 720 | 0.0066 | 0.00172 | 690.0 | | | | |
| 1,200 | 0.007 | | | | | | |
| 2,000 | 0.007 | | | | | | |
| Average..... | | 0.00186 | | | | | |

[4] The word protein is used in this paper as a convenient expression to signify either protein or any other amphoteric electrolyte, which acts like protein in combining with dyes. *Cf.* Foot-note 5. It is quite possible that there are several proteins or other amphoteric electrolytes in the cell which combine with the dye.

It might be thought that the dye when combined with the protein would have a different color and that in consequence the apparent concentration would differ from the concentration as measured. It was found, however, that on mixing dye with cell sap the change of color was the same as on mixing with an equal amount of water. It may therefore be assumed that this source of error is negligible.

[5] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

protein which cannot combine with the dye). This ionized protein must be negatively charged because the dye with which it combines is basic and its colored ion is positively charged.

As the amount of active protein increases, more dye is able to combine. The process may be represented as follows:

$$I \rightarrow A$$

$$A + D = AD$$

where $I$ = inactive protein, $A$ = active protein, and $D$ = dye (cation).

If we assume that the transformation of $I$ to $A$ takes place rapidly (as compared with the second reaction) we need, in our explanation of the time curve, to consider only the latter, $A + D = AD$. Since the amount of $D$ is constant (because it is present in great excess) we may regard the reaction as monomolecular, just as it is in the case of the inversion of cane-sugar in the presence of great excess of water.

The concentration of the dye in the external solution remains constant during the reaction because it is present in great excess. The question arises whether this is also the case inside the cell where the reaction $A + D = AD$ takes place. Since the temperature coefficient of the whole process is high it would appear that the chemical reaction is slower than the process of diffiusion (if the latter were slower we should find a low temperature coefficient). Under these circumstances we may assume that the concentration of $D$ would not diminish as the reaction proceeded, and if it increased the amount of increase could not be very great during the period of the experiment which is employed in our calculations and could in no case go above 0.002 per cent (the concentration in the external solution). It therefore appears that we cannot go far astray in assuming it to be constant.

## IV.

### *Penetration of Dye Accompanied by Injury.*

When we apply this explanation to the curves at pH values higher than 7.0 (Fig. 2), we meet with a complication owing to the fact that at these higher pH values some injury occurs after a time (as indicated by the fact that chlorophyll granules appear in the sap when it is squeezed out of the cell). This seems to be due to the

accumulation of the dye in the vacuole and to the effect of the buffer solutions. For example, the death of the cells takes place in about 5 hours, when they are placed in the buffer solution[6] at pH 9, while it takes place in about 4 hours in the solution of dye at pH 9, when the concentration of the dye in the sap reaches about 0.2 per cent, as shown in Fig. 3. As a result of this injury, the cell dies before an equilibrium is reached. If the injury goes far enough the accumulation of the dye stops and the dye begins to diffuse out of the cell very rapidly.

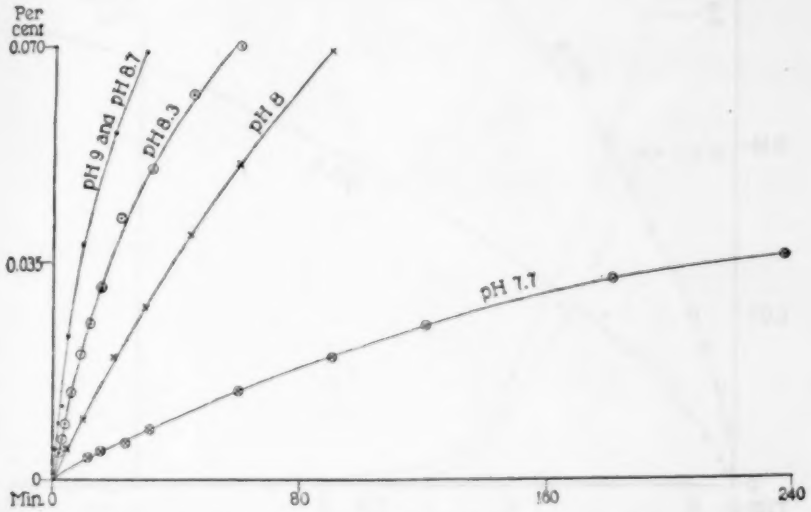


FIG. 2. Curves showing the penetration of 0.002 per cent cresyl blue solution into living cells of *Nitella*, at pH 7.7, 8, 8.3, 8.7, and 9. The concentrations (in per cent) of the dye in the cell sap are plotted as the ordinates, and the time in minutes as the abscissæ.

In dealing with the curves for pH values above 7.0 I have disregarded that part of the curve which is affected by this injurious action. In order to be on the safe side I have disregarded these portions which lie above 0.04 per cent.

If the reaction $A + D = AD$ behaves as one of the first order (since $D$ is practically constant), its velocity constant will remain the same no matter how much the initial concentration of $A$ varies.

[6] The effect of the buffer solutions in the cells will be discussed later in this paper.

It will therefore be the same for all values of pH (if the chemical nature of $A$ does not change with change of pH). We find that this is true for pH 6.6 and 7.0 where the equilibria are known, and we assume that it is true for the higher values where the equilibria cannot be precisely determined (owing to the onset of injury).[7] We therefore assume that the value of $K$ is in all cases 0.00184 (average of 0.00182 and 0.00186) and calculate the equilibria at each pH on

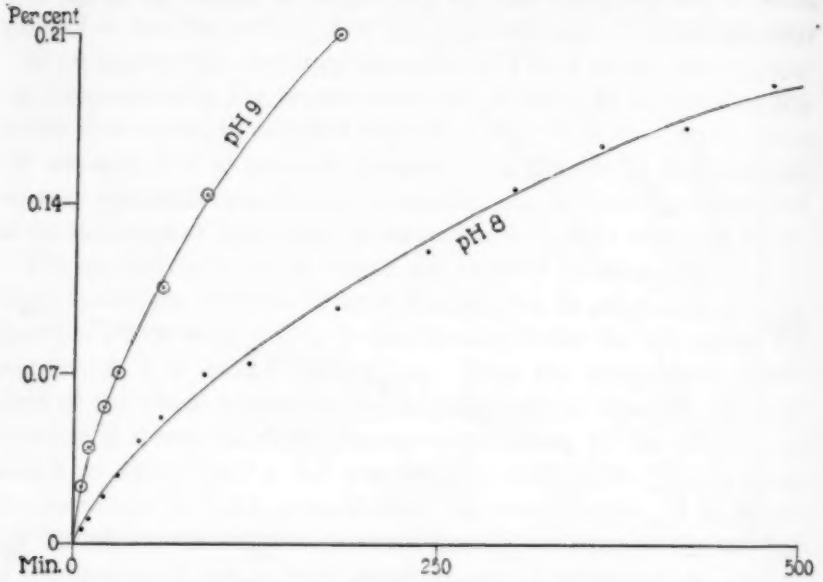


FIG. 3. Curves showing the penetration of 0.002 per cent cresyl blue solution at pH 8 and pH 9, into living cells of *Nitella* until the death of the cells takes place. The concentrations (in per cent) of the dye in the cell sap are plotted as the ordinates, and the time in minutes as the abscissæ.

this basis. When this is done we find that the values of $K$ are approximately constant[8] at 0.00184 throughout each curve.

[7] It might be possible to fit these curves with a formula depending on consecutive reactions, but since injury seems to be involved and we are therefore dealing with variables which are not directly measured, it seems unnecessary to carry out such calculations.

[8] Above 0.04 per cent concentration of the dye in cell sap a decrease in the velocity constants takes place, which is in all probability due primarily to the injury.

On comparing the equilibria obtained at different pH values, it is found that the equilibrium increases with increase[9] in pH, as shown in Fig. 4, Curve E, where the equilibria are plotted as the ordinates, and pH values as the abscissæ.

If it is possible for the chemical nature of the active protein to differ at different pH values, and if this should be true in the present instance, the same velocity constants may not be maintained for

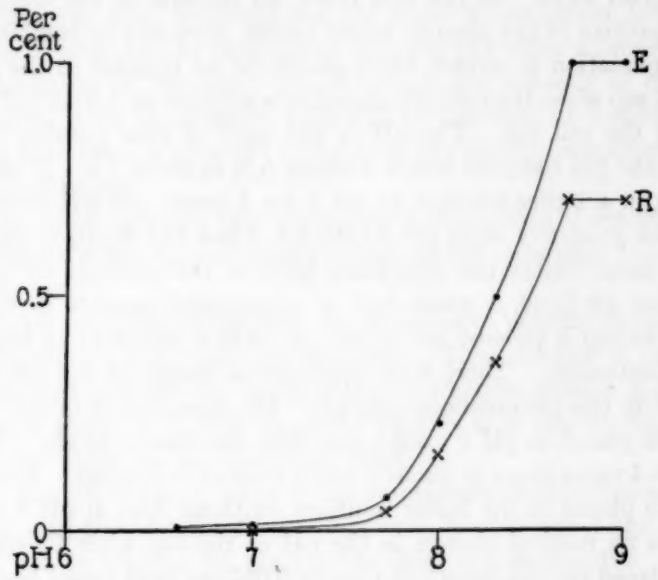


FIG. 4. Curves showing the penetration of 0.002 per cent cresyl blue solution at different pH values into living cells of *Nitella*. In Curve E, the equilibria are plotted as the ordinates, and pH values as abscissæ. In Curve R, the ordinates represent rates, (reciprocals of the times required to bring the concentration of the dye in cell sap to 0.006 per cent).

[9] The rate of penetration of dye was found for each pH by obtaining the reciprocal of the time it took the dye to reach the concentration of 0.006 per cent in the cell sap. This rate increases with the increase in pH as shown in Curve R, Fig. 4.

This seems to be true for all the basic dyes employed. Experiments with acid dyes show that they do not penetrate even at low pH values (pH 4.0); experiments at lower pH values are not feasible owing to the rapid injury which occurs. It seems probable that pH 4 does not lie far enough below the isoelectric point of the protoplasm to provide active protein which can combine with the acid dye.

all pH values; in such a case our assumption might have to be modified, but this may not alter the main conclusion that the equilibrium rises with the increase in pH value.

V.

*Presence of Active Protein in the Cell.*

The active protein might influence the equilibrium reached by the dye in two ways. In the first place, an increase in the amount of active protein in the vacuole would enable more dye to combine. If this explanation is correct, there should be an increase in the pH of the cell sap when the cells are placed in a solution at higher pH than that of the cell sap. The pH of the sap[10] of cells growing in tap water (the pH value of which is about 6.5) is about 5.6. If the cells are left in a buffer solution at pH 9 for 5 hours, the pH of the sap increases gradually from pH 5.6 to 6.5, when the death of the cells takes place. After the cells have been in the solution for a little less than an hour, a great deal of chlorophyll appears in the sap when the sap is pressed out of the cell (which indicates an injury in many instances). There is no appreciable change in the pH of the sap before this phenomenon appears. The same is true in the case of the cells placed in pH 8 except that here the change in the pH from 5.6 to 6.4 takes place in about 9 hours instead of 5 hours. When the cells are placed in the buffer solutions (without dye) at pH 8 and 9, there is no marked change in the pH of the sap after 10 minutes. Cells placed for this length of time in 0.002 per cent cresyl blue solution would show a concentration in the sap of 0.04 per cent at pH 9, and 0.012 per cent at pH 8. These experiments show that there is a considerable difference in the amount of dye combined at pH 8 and 9, and yet there is no appreciable change in the pH of the sap. With the present method, however, it is not possible to detect a change in pH of the sap less than 0.1 pH so that a difference of less than 0.1 pH must bring about the above difference in the amount of dye combined at pH 8 and 9.

[10] To determine the pH value, sap was drawn into a capillary tube to a certain height and blown out on to a slide; nine parts of sap were mixed with one part of of a very dilute solution of brom-cresol purple which was measured in the same way. The color was then compared with a mixture of nine parts of buffer solution and one part of the same indicator.

We may therefore conclude that if the increase in the active protein in the vacuole is responsible for the increase in equilibrium, a small increase in the pH of the cell sap suffices to bring about a considerable change in the amount of active protein.

It is quite possible, however, that this is not a fair comparison since the increase in the pH of the sap with the same external pH may be greater when the dye is present in the external solution. The cell sap contains organic acids, and if the dye cation penetrates in combination with hydroxyl ion (as some investigators believe), the organic acids will be neutralized and the pH value increased by the penetration of the dye. (In that case the amount of dye in combination with the organic acid will be constant at equilibrium and will thus form a constant fraction of the total concentration of dye at equilibrium.)

It is, of course, possible that the sap near the surface of the vacuole may undergo a much more rapid change in pH and that this may affect the result.

Since this explanation assumes the presence of protein in the sap some experiments were made on this point. The presence of protein in the vacuole is shown by the fact that it contains protein globules which float about in it, and dissolved protein is shown by the fact that the sap gives the xanthoprotein reaction.

When the cell is killed, some of the amphoteric electrolytes appear to diffuse out; the protein globules, however, remain intact and these globules can be stained by placing the dead cell in the dye. It is found that these globules stain more rapidly at high pH than at low pH. This agrees with the experiments on the penetration of dyes into the cells.

In the second place, it is of course, possible that the increase in the amount of dye taken up is due to changes in the protoplasmic layer. Since this is very thin it might undergo rapid changes in pH with a consequent increase in active or ionized protein. If the protoplasmic layer takes up the dye and the dye is actively driven into the vacuole it is quite conceivable that the rate and the final equilibrium of this process would depend on the amount of active protein present. Such a process would involve the expenditure of energy derived from the physicochemical activity of the cell.

## VI.

## DISCUSSION OF LITERATURE.

Some investigators[11] suggest that the basic dye penetrates more rapidly at high pH because the dye cation can enter only when combined with OH, and that more of this compound, which will be called DOH, is formed at the higher pH values. If this were the case it could affect only the rate of penetration (in the case of *Nitella*) since the final equilibrium would depend only on the amount of substance combining with the dye. If it affected the rate it would change our assumption that the velocity constants are the same at each pH but it would not affect our conclusion that the final equilibrium increases with increase of pH.

If the dye did not combine with something in the cell the concentration inside could not rise above that of DOH. If the dye combines with something inside the cell this combining substance will determine the final equilibrium.

Harvey maintained that the dye accumulated because the dye was unable to diffuse out from the sap, because the sap was acid, which decreased the amount of DOH. If this is true there should not be a diffusion of dye from the cell sap when the stained cells are placed in a buffer solution of low pH. But experiments[1] show that the dye diffuses out much more readily at low pH than at high pH, so that neither the penetration nor the accumulation can be dependent primarily on the amount of DOH.

The solubility of basic dye in fatty substances is known to increase in alkali.[12] If the plasma membrane were lipoid, as some investigators suppose, the basic dye could diffuse out of the cell more readily at high pH than at low pH, but my experiments show that the dye comes out more readily when the pH value of the external solution is low.

Pfeffer[13] states that a basic dye forms an insoluble compound with

[11] Harvey, E. N., *J. Exp. Zool.*, 1911, x, 508.

[12] Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1.

[13] Pfeffer (Pfeffer, W., *Untersuch. Bot. Inst. Tub.*, 1886, ii, 179) supposes that in some cases the dye forms soluble compounds in the sap which are unable to diffuse out. Various substances such as protein, phloroglucin, etc., have been supposed to combine with the dye in some cases.

tannic acid in the sap of *Spirogyra*, for which reason accumulation of the dye takes place. If this compound is the primary factor in the process, the equilibrium should be the same at all pH values, provided the tannic acid is not neutralized by anything except DOH, as would be the case if the cell is impermeable to alkali. If the cell is permeable to alkali it will diffuse in and compete with the DOH for the tannic acid, in which case the dye equilibrium will be decreased as the external pH value increases.

MacArthur[14] showed that planarians took up more basic dye at high pH than at low pH. He stated that he was unable to determine how much of this effect was due to the influence of hydrogen ion concentration on dissociation or rate of diffusion of the dyes, and how much to the alteration of "membranes" and "deeper tissues."

Bethe[15] has drawn an analogy between the vital staining and staining of protein with dyes at different hydrogen ion concentrations. In both cases, there was a greater staining with a basic dye at high pH, and with an acid dye at low pH.

Redfern[16] states that different equilibria were reached at different concentrations, when discs of vegetable tissue were placed in the solutions of basic dye. She concluded that the process of staining was due to adsorption. Since her method differs greatly from mine, I shall be obliged to defer the comparisons to the future.

A theory[11,15,17] of the penetration of dye into the cells must be reserved for the future when greater knowledge of the facts may solve some of the complications. I feel, however, that the final equilibrium is determined by the amount of active protein present. Whether this is dependent primarily on the protein in the cell sap, or

[14] MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350.

[15] Bethe, A., *Biochem. Z.*, 1922, cxxvii, 18. For other recent papers on the influence of pH on vital staining see Rohde, K., *Arch. ges. Physiol.*, 1920, clxxxii, 114. Pohle, E., *Deutsch. med. Woch.*, 1921, xlvii, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, lx, 354.

[16] Redfern, G. M., *Ann. Bot.*, 1922, xxxvi, 511.

[17] For reviews of the literature on the penetration of dyes, see Bayliss, W. M., Principles of general physiology, New York, London, Bombay, Calcutta, and Madras, 1915. Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic and Berlin, 1914. Overton, E., *Jahrb. wissensch. Bot.*, 1900, xxxiv, 669. Schulemann, W., *Biochem. Z.*, 1917, lxxx, 1.

in the protoplasm (either at the surface or throughout the protoplasm), must remain undecided for the present. If it is dependent on the protein in the cell sap, a small change in the pH must bring about a considerable change in the amount of active protein. If, on the other hand, it is dependent on the protein in the protoplasmic layer (the pH of which is assumed to change rapidly), the dye must be driven into the cell sap from the protoplasm by the energy supplied by the physicochemical processes in the cell.

## VII.

## SUMMARY.

When cells of *Nitella* are placed in buffer solutions at pH 9, there is a very slow and gradual increase in the pH of the sap from pH 5.6 to 6.4 (when death of the cells takes place). If the living cells are placed in 0.002 per cent dye solutions of brilliant cresyl blue at different pH values (from pH 6.6 to pH 9), it is found that the rate of penetration of the dye, and the final equilibrium attained, increases with increase in pH value, which can be attributed to an increase in the active protein (or other amphoteric electrolyte) in the cell which can combine with the dye.

# COMPARATIVE STUDIES ON RESPIRATION.

## XXVII. The Mechanism of Oxidation in Relation to Chloroform Anesthesia.

By GEORGE B. RAY.

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(Received for publication, May 21, 1923.)

The preceding papers (1) in this series have shown that chloroform produces similar effects on the rate of production of $CO_2$ by living and killed tissue and by unsaturated organic acids. In the case of dead tissue and organic acids, the system consisted of oxidizable material, $H_2O_2$ and $Fe_2(SO_4)_3$. The experimental results indicate that similar processes are taking place in all cases. In this paper an attempt is made to verify this statement and to show that a mathematical interpretation of the curves indicates that the reactions are all of the same order.

There are certain considerations that must be taken into account in order to derive a formula which will approximate the conditions found experimentally. It is necessary to assume that there is present in the reaction mixture, before the addition of chloroform, a complex consisting of two, or possibly three, of the components of the reaction. It is plausible to assume that this condition exists in the reaction, for it is quite evident that here we have to deal with an "induced reaction." Mellor (2), in discussing this type of reaction, points out that it may be considered as taking place in two ways; either that the reaction is catalyzed by the presence of the third reacting substance, which may mean the formation of a complex, or that two (or possibly three) of the constituents of the reaction combine.

In the case which we are discussing—oxidation by means of $H_2O_2$ and $Fe_2(SO_4)_3$—we consider that the complex is being broken down at a constant rate, with a production of $CO_2$ directly proportional to the rate of decomposition of the complex. We assume that the reaction starts from a substance, $A$, which in turn goes into the com-

plex ($X$), which is the factor concerned with the production of $CO_2$; and that $X$ upon disintegration is transformed into $B$, which is non-reactive.

The addition of chloroform introduces a fourth factor into the reaction with the result that the changes taking place in the complex are definitely affected. Such a condition may exist if the chloroform acts as a catalyst. At the same time chloroform need not be considered to be entirely indifferent chemically for it is possible that it may unite with certain substances to form addition compounds. This is indicated by the work of Moore and Roaf (3) who worked with serum, and by that of Krüger (4) in the case of hemoglobin. If the action of the chloroform is catalytic in nature it does not preclude the conception that there is a loose combination with some constituent of the system.

The curve of the rate of production of $CO_2$ after the addition of chloroform is very much like the curve found by Rutherford and Soddy (5) for the decay of thorium compounds. These authors found that when the activity of a thorium compound is plotted against time, the resulting curve rises and falls just as in the case of the action of chloroform on the production of $CO_2$. If we assume that the reactions $A \rightarrow X$ and $X \rightarrow B$ are monomolecular (and irreversible, or practically so) we may apply the formula developed by Rutherford and Soddy. This formula is

$$X_1 = \frac{A_0 k_1}{k_2 - k_1} \left( e^{-k_1 T} - e^{-k_2 T} \right)$$

where $X_1$ is the concentration of the complex, $X$, produced from $A$ during the time, $T$, after the addition of the chloroform; $k_1$ is the velocity constant of the reaction, $A \rightarrow X$, and $k_2$ of the reaction, $X \rightarrow B$; and $e$ the base of natural logarithms. This formula gives the quantity of $X$ without taking into consideration the amount present at the instant the chloroform was added. The amount of $X$ present at this time diminishes (by the reaction $X \rightarrow B$) after the chloroform is added; if no formation of $X$ occurred the amount remaining at the time, $T$, would be

$$X_0 = e^{-k_2 T}$$

where $X_0$ is the amount of $X$ at the instant the chloroform was added, and $T$ is the time which has elapsed since the addition of the chloroform.

To represent the whole processs it is necessary to combine the formulæ given above. In this case let $X_T$ be the total amount of $X$ present in the solution at the time, $T$. Since we have given the experimental data in terms of per cent it will be necessary to multiply the value of $X_T$ by a constant and to add a constant when the curve does not fall to zero. These constants will vary with the type of material used in the experiments.

The final formula will be

$$X_T = \left[X_0 e^{-k_2 T} + \frac{A_0 k_1}{k_2 - k_1}\left(e^{-k_1 T} - e^{-k_2 T}\right)\right] K + K'$$

This formula is practically the same as the one developed by Osterhout (6) for the resistance of living tissue.

In the calculations which follow it is assumed that at equilibrium (before the addition of chloroform) the ratio of $X_0$ to $A_0$ is as 1 to 30, and that the value of $A_0$ is 9. Therefore, the value of $X_0$ will be 0.3. The values of the constants $K$ and $K'$ are determined as follows: The base line of the curve is taken as the point at which the rate of production of $CO_2$ becomes constant. The difference between this and the normal rate of production of $CO_2$ (which is taken as 100 per cent) is divided by 0.3, thus resulting in the value of $K$. $K'$ is the difference between the base line of the curve and the time axis.

The application of this formula to the experiments published in the previous papers shows some very interesting results.

Fig. 1 shows a curve obtained by averaging the curves which show the effect of chloroform on the rate of production of $CO_2$ by oleic, fumaric, and cinnamic acid. The calculated curve is shown as a broken line. The constants used in this case are $k_1 = 0.05$, $k_2 = 0.5$, $K = 133$, and $K' = 60$.

Fig. 2 gives the curves for the rate of production of $CO_2$ from killed *Ulva* in the presence of $H_2O_2$ and $Fe_2(SO_4)_3$, as compared with the calculated curves. The broken lines are the calculated curves and the solid lines the experimental. Curve A represents the effect of 1 per cent chloroform when 10 cc. of 0.002 M $Fe_2(SO_4)_3$ are present.

The constants for the calculation are $k_1 = 0.256$ and $k_2 = 2.5$. Curve B represents the effect of chloroform when 20 cc. of the iron solution are used. The constants in this case are $k_1 = 0.02$ and $k_2 = 1.00$. In both instances the values of $K$ and $K'$ are 200 and 50, respectively.

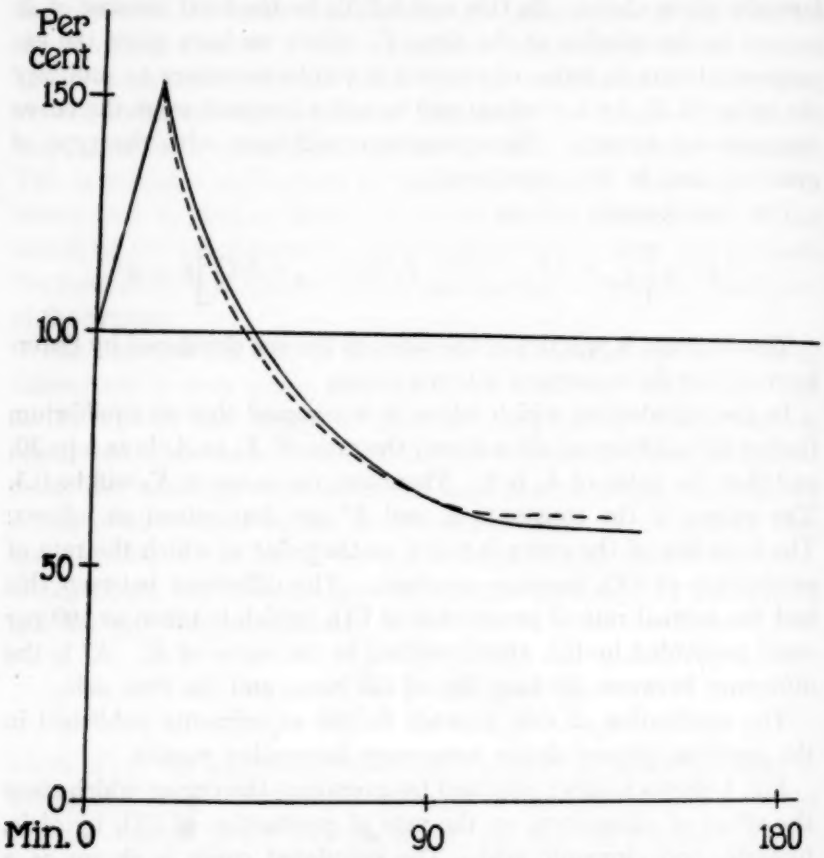


FIG. 1. The effect of chloroform on the rate of production of $CO_2$ by cinnamic, oleic, and fumaric acids. The solid line is the average curve of the experiments with these acids. The broken line is the value of $X_T$ at various times ($T$) when the velocity constants are $k_1 = 0.05$ and $k_2 = 0.5$. $K$ and $K'$ are 133 and 60, respectively.

Fig. 3 gives the experimental and the calculated curves for the action of chloroform on the rate of production of $CO_2$ by living *Ulva*. A is the curve for the action of 0.5 per cent chloroform, and B the

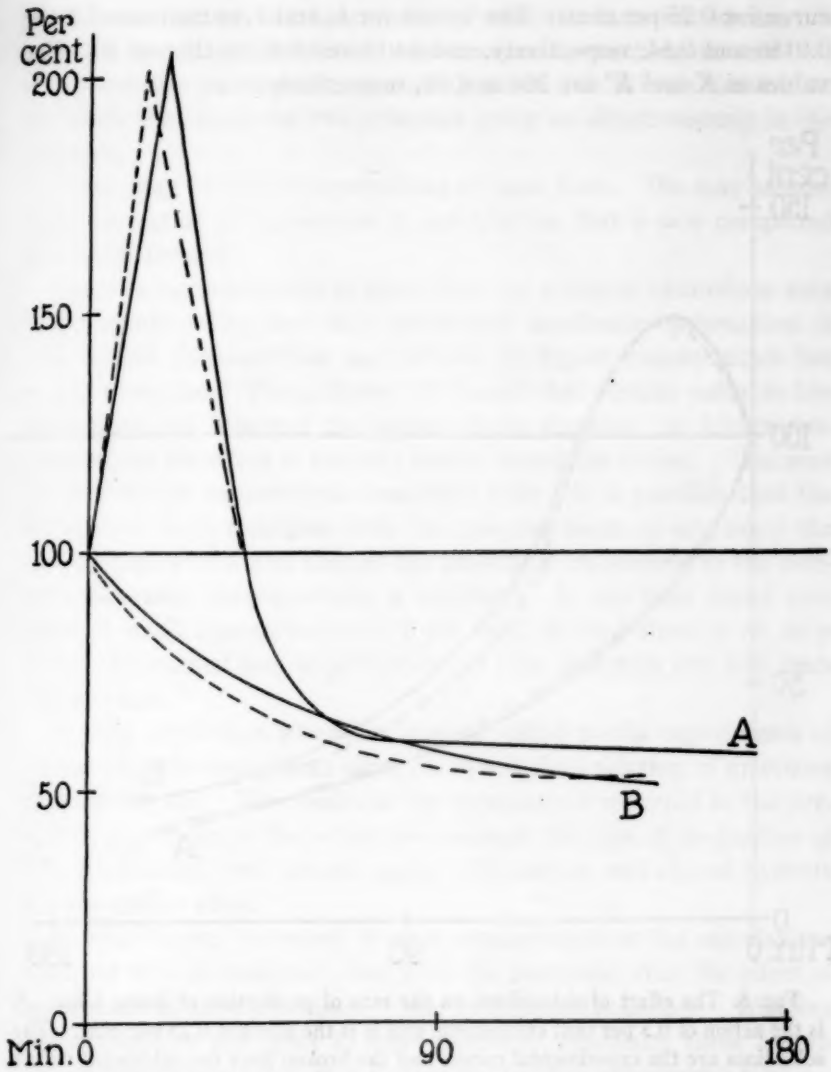


FIG. 2. The calculated (broken line) and the experimental (solid line) curves of the effect of chloroform on the rate of production of $CO_2$ by killed *Ulva* when treated with $H_2O_2$ and $Fe_2(SO_4)_3$.

*A*. 10 cc. of iron. $k_1 = 0.256$, $k_2 = 2.5$, $K = 200$, and $K' = 50$.
*B*. 20 " " " $k_1 = 0.02$, $k_2 = 1.00$, $K = 200$, " $K' = 50$.

curve for 0.25 per cent. The values for $k_1$ and $k_2$ in the case of A are 0.0186 and 0.54, respectively, and 0.018 and 0.4, in that of B. The values of $K$ and $K'$ are 266 and 20, respectively.

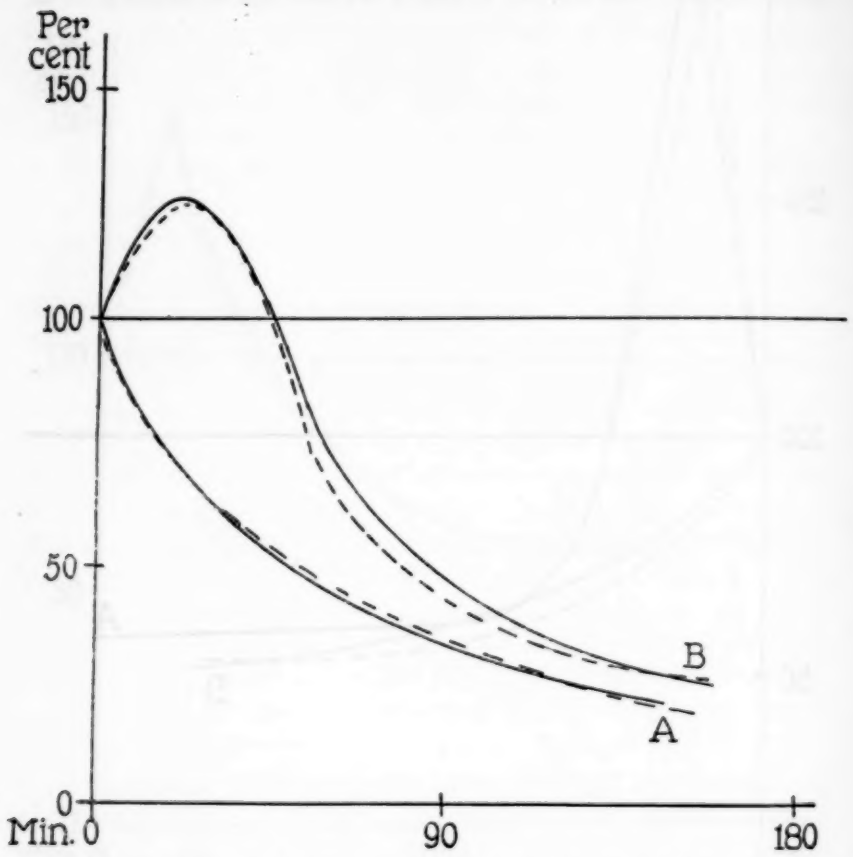


FIG. 3. The effect of chloroform on the rate of production of living *Ulva*. A is the action of 0.5 per cent chloroform, and B is the effect of 0.25 per cent. The solid lines are the experimental curves and the broken lines the calculated values of $X_T$. In A, $k_1 = 0.0186$ and $k_2 = 0.54$. In the case of B, $k_1 = 0.018$, and $k_2 = 0.4$. In both A and B, $K = 266$ and $K' = 20$.

As may be seen from the figures, there is a close agreement between the calculated and the experimental curves. This would seem to indicate that the same process is taking place in each case. At the

same time it must not be considered that the fact that we have a formula based on consecutive monomolecular reactions means that these are the exact processes taking place. It is only feasible to conclude that there are two processes going on simultaneously in the reaction.

There may be two interpretations of these facts. We may assume that the action of chloroform is catalytic or that a new compound has been formed.

There is some evidence to show that the action of chloroform may be catalytic. The fact that chloroform accelerates production of $CO_2$ in low concentration and hinders at higher concentration has many analogies. Thus, Hoyer (7) found that certain acids in low concentrations hastened the action of the enzyme. In higher concentrations the effect of the acid was to retard the action. This may be true in the experiments considered here. It is possible that the chloroform as it combines with the complex tends to accelerate the production of $CO_2$, and that as the amount of chloroform in the complex increases, decomposition is inhibited. It has been found that when a small concentration (0.5 per cent) is used there is no drop below the normal rate of production of $CO_2$, although one still finds the increase.

In this connection attention may be called to the experiments of Acton (8), who found that ether catalyzed the oxidation of arsenious acid by the air. The results of the experiments reported in the preceding papers show that ether can increase the rate of production of $CO_2$ from tannic and fumaric acids. Chloretone and chloral hydrate have a similar effect.

It would seem, therefore, from a consideration of the calculations together with the evidence cited from the literature, that the effect of chloroform in these experiments may be chemical in nature. Whether this conception will apply to other narcotics, especially those known as indifferent narcotics, is a matter for further experimentation.

It is evident that certain widely accepted theories of anesthesia fail to explain these results. It does not seem probable that lipoid solubility could play a part in this reaction since it has been shown that the process will take place in solutions where lipoids are absent. Such is also the condition in regard to the possible effect on the

colloids. It has also been considered that narcotics owe their action to the changes produced in the membranes of the cell. Without doubt this may play a part in certain life processes, but the effect of chloroform on the oxidative system of the cell seems to be concerned directly with that system and to be distinct from the effects on permeability.

## SUMMARY.

A mathematical analysis of the effects of chloroform on the production of carbon dioxide by living *Ulva*, killed *Ulva*, and unsaturated organic acids indicates that the same process is taking place in all three cases. On the basis of this interpretation it is reasonable to conclude that the action of chloroform on the oxidative mechanism of the cell is chemical in nature and that it acts either by catalysis or by the formation of a loose compound with some portion of the system.

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# NOTE ON THE PURIFICATION AND PRECIPITATION OF CASEIN.

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(Received for publication, May 16, 1923.)

The numerous methods for the preparation of casein all make use of the fact that casein may be precipitated at its isoelectric point by the addition of acid to an alkaline solution of the protein. It is also known that in the presence of strong acid the protein is "denatured" and becomes permanently insoluble. Great care must be used therefore in the addition of acid. This point was recognized by Van Slyke and Baker,[1] who added the acid slowly with very rapid stirring. The method, however, required very careful handling, and even under the best conditions small amounts of denatured casein are formed, as can be seen from the cloudy solution obtained with alkali. Since it is the acid which causes the formation of this denatured casein, it would appear more logical to make the final precipitation with alkali, since small excess of alkali does not affect the protein. This procedure was tried and was found to yield a product free from denatured casein. The method adopted is briefly as follows.

Casein is precipitated from milk as described by Van Slyke and Baker, except that it is not necessary to take unusual precautions with the addition of acid. The precipitate is thoroughly washed to remove any soluble salts. About 10 gm. of the precipitate are then suspended in a liter of water and the suspension brought to a pH of 2.5 to 3.0 by the addition of HCl. A cloudy solution results. This is then filtered through hardened fluted filter paper, if necessary, several times. A perfectly clear filtrate is obtained which shows only a slight Tyndall cone, and which contains the casein. The filtration is very slow and may require several days, but no other method could

[1] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

be found to remove the very fine particles of denatured protein. This solution is then brought to pH 4.7 by the addition of NaOH. The precipitate of casein is washed several times with distilled water and either dried with acetone or kept as a fine suspension under toluene.

Casein prepared in this way gives an almost water-clear solution in either acid or alkali. If, however, the clear alkaline solution is precipitated by the addition of acid, a milky suspension results which cannot be centrifuged or filtered except in a very narrow range of pH near 4.7. It is possible, therefore, to have two entirely different solutions of casein in the pH range of 5.0 to 7.0 and from 4.5 to 3.0, depending on whether the acid or alkali was added to the precipitate of isoelectric casein or to the solution of casein. The suspension prepared from the precipitate by the addition of alkali consists of a perfectly clear liquid which may be easily filtered or centrifuged from a flaky white precipitate. If the clear alkaline solution is partly precipitated by acid, however, a milky liquid is obtained from which the solid casein cannot be separated by any of the ordinary procedure. This difference is due to the protective action of the casein in solution. As soon as a particle of isoelectric (or denatured) casein is precipitated in the solution, it is covered by a film of the soluble casein salt and therefore the particles do not coalesce. As the isoelectric point is approached, however, the concentration of the soluble casein becomes reduced, and near pH 4.7 becomes so small that it is no longer sufficient to protect the particles which therefore coalesce into large flakes. When, on the other hand, alkali is added to the isoelectric casein, some alkali salt is formed and dissolves. The presence of this salt cannot break up the large flakes of isoelectric casein in suspension, although, as was stated above, it can prevent their formation. The solution of isoelectric casein in alkali is a purely solubility effect, as stated by Loeb,[2] whereas the precipitation of casein from such solutions is complicated by the fact that the solid casein formed is kept in very fine suspension by the protective action of the soluble casein salt.

[2] Loeb, J., and Loeb, R. F., *J. Gen. Physiol.*, 1921–22, iv, 187. Cohn and Hendry (Cohn, E. J., and Hendry, J. L., *J. Gen. Physiol.*, 1922–23, v, 521) have shown conclusively that the solubility of casein in alkali is true solubility governed by the solubility product.

# THE INACTIVATION OF TRYPSIN.

## IV. The Adsorption of Trypsin by Charcoal.

By JOHN H. NORTHROP.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, May 19, 1923.)

It has been frequently observed that trypsin is more or less completely removed from solution by charcoal. The reaction has been studied in detail by Hedin[1] who found that the amount removed depended on the time of standing, the order in which the components of the solution were mixed, and, in general, behaved as a typical heterogeneous reaction. Hedin also studied the inhibiting action of serum on trypsin and concluded that it was analogous to the action of charcoal. Hussey and the writer[2] have found, however, that the reaction between the inhibiting substance and trypsin could be accurately calculated by the laws of homogeneous reactions. The reaction was found to be completely and instantly reversible and independent of the order in which the components were mixed. The discrepancy between these results and those obtained by Hedin is due to the fact that Hedin allowed the mixture of trypsin and plasma to stand some time before measuring the amount of trypsin combined. Under these conditions the trypsin undergoes a secondary irreversible inactivation which complicates the results. It is quite true that under these conditions the order of mixing, etc., plays an important part, as Hedin found. This is due, however, as the writer showed[3] in the case of the equilibrium between trypsin and the inhibiting substance (formed by its action on proteins), to a secondary irreversible inactivation of the trypsin and is not a property of the reaction between the trypsin and

[1] Hedin, S. G., *J. Physiol.*, 1904–05, xxxii, 390; *Z. physiol. Chem.*, 1906–07, l497; *Biochem. J.*, 1906, i, 484; 1907, ii, 81; *Ergebn. Physiol.*, 1910, ix, 433.

[2] Hussey, R. G., and Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 335.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 261.

the inhibiting substance. In order to confirm this explanation and to be sure that the reaction with charcoal differs from that with serum and the other inhibiting substances studied, it seemed important to repeat the experiments with charcoal under the same conditions that were used in the experiments with serum. The present paper is a report of these experiments. The results obtained with charcoal confirm those of Hedin, but are qualitatively different from those with serum. There is no analogy therefore between the two reactions and therefore no reason for considering the reaction with serum as heterogeneous.

*Experimental Methods.*

The trypsin was determined, as described by Hussey and the writer,[4] by noting the time required to cause a 10 per cent change in the viscosity of a standard gelatin solution. The charcoal used was a preparation manufactured by the General Chemical Company and labelled "decolorizing charcoal." It was finely ground and washed with water and acetone before use.

A 2 per cent solution of Fairchild's trypsin in glycerin was used as a stock solution. It was diluted 20 times with water or buffer solution.

The relation between the amount of trypsin removed from solution and the quantity of charcoal is shown in Fig. 1. The curve is of the general form obtained either in adsorption reactions or with reversible homogeneous equilibria. It can be calculated fairly well on either basis but since the adsorption formula is without theoretical interest and the reaction cannot be considered as a reversible equilibrium, it is unnecessary to give the figures. The experiment shows, however, that very little can be deduced from the results of experiments in which the amount of one of the components alone is varied. The results, as a rule, can be calculated either as homogeneous or heterogeneous reactions. The reversibility of the reaction is a much more useful test. Heterogeneous reactions, as a rule, are slowly and *incompletely* reversible, whereas homogeneous equilibria, and especially ionic reactions, are rapidly and completely reversible. As was stated above, the

[4] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922–23, v, 353.

reaction between trypsin and the inhibiting substance in serum is rapidly and completely reversible, while the reaction with charcoal is not.

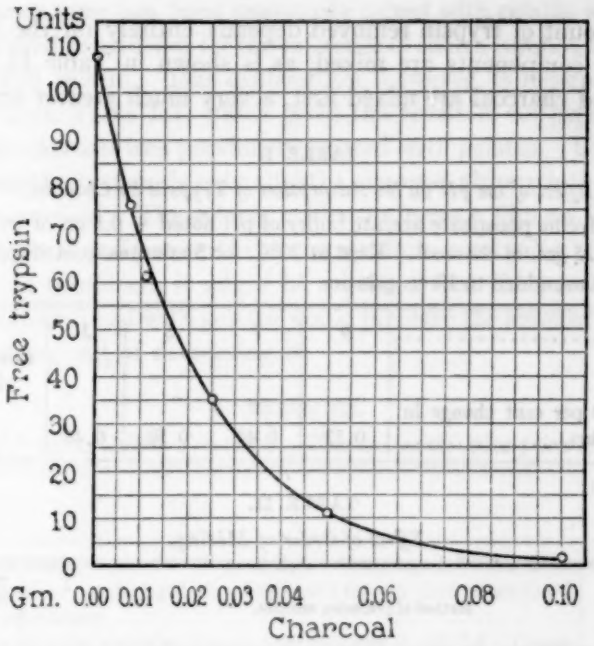


FIG. 1. The effect of increasing amounts of charcoal on the removal of trypsin from solution. 5 cc. of a 0.1 per cent trypsin solution in glycine phosphate acetate buffer pH 8.0 added to the amounts of charcoal indicated. Kept 0.5 hour at 25° C., centrifuged, and trypsin determined in 0.2 cc. of the supernatant liquid.

*Effect of the Acidity of the Solution.*

The result of an experiment at various pH is given in Table I. The amount of trypsin combined with charcoal is evidently independent of the pH within a wide range. This is entirely different from the results obtained when pepsin combines with solid protein.[5] Unpublished experiments show that trypsin also combines with solid protein and that the combination is markedly affected by the pH.

[5] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113; 1920–21, iii, 211.

The mechanism is apparently entirely different from the reaction with charcoal and is closely connected with the Donnan equilibrium.

*Effect of the Order of Mixing the Components.*

The amount of trypsin removed depends entirely on the order in which the components are mixed, as is shown in Table II. If the trypsin and charcoal are mixed first, a very much greater amount of

TABLE I.

*Effect of the pH on the Adsorption of Trypsin by Charcoal.*

5 cc. of glycine phosphate acetate buffer of pH noted + 0.5 cc. of trypsin solution + 0.0125 gm. of charcoal. Kept at 20°C. for 5 minutes, centrifuged, and 0.2 cc. supernatant added to 10 cc. gelatin.

| pH | 9 | 7 | 5 | 3 | 7 Control, no charcoal. |
|---|---|---|---|---|---|
| Time for 10 per cent change in viscosity, hrs. | 0.52 | 0.49 | 0.50 | 0.48 | 0.15 |

TABLE II.

*Effect of Order of Mixing.*

| Method of preparing mixture. | Time required for 10 per cent change in viscosity. |
|---|---|
| | *hrs.* |
| 1. 1 cc. of $H_2O$ + 0.2 cc. of trypsin, kept 5 min. at 20°C., 10 cc. of gelatin added | 0.41 |
| 2. 1 cc. of charcoal suspension (= 0.1 gm. of charcoal) + 0.2 cc. of trypsin, kept 5 min. at 20°C., 10 cc. of gelatin added | >3.00 |
| 3. 10 cc. of gelatin + 1 cc. of charcoal suspension kept 5 min. at 20°C., 0.2 cc. of trypsin added | 0.42 |

the enzyme is combined than if the charcoal is first mixed with the gelatin. This is typical of a heterogeneous reaction and contrary to most homogeneous equilibria. It is exactly the opposite of the results obtained with serum or other inhibiting substances when the experiment is carried out under the same conditions.

The experiment also shows that the combination is not reversible and that the trypsin combined with charcoal is inactive.

It might be supposed that this result is due to the fact that the trypsin is combined with the gelatin in conformation with the usual hypothesis of an intermediate compound between substrate and enzyme. The failure of the trypsin to combine with the charcoal when the enzyme has been previously mixed with gelatin would then be considered as evidence for the existence of such a compound between trypsin and gelatin. This explanation, however, is incorrect as may be seen from Table III, which gives the result of an experiment in which the *charcoal* was previously treated with gelatin. Under these conditions the trypsin is only slightly removed although the charcoal had been washed. The gelatin evidently forms a film on the surface of the charcoal and prevents the trypsin from combining. If trypsin and gelatin combined, it might be expected that the previous treatment of the charcoal with gelatin would increase the amount of trypsin combined instead of decreasing it.

TABLE III.

*Effect of Previous Gelatin Treatment of Charcoal on the Adsorption of Trypsin.*

| | Units of trypsin per 0.2 cc. of solution. |
|---|---|
| 1. 0.1 gm. of charcoal added to 10 cc. of $H_2O$. Centrifuged, 5 cc. of dilute trypsin added; centrifuged after 5 min. and trypsin determined in 0.2 cc. of supernatant | <0.10 |
| 2. 0.1 gm. of charcoal added to 10 cc. of 3 per cent gelatin pH 7.4. Centrifuged, washed with 10 cc. of $H_2O$, and trypsin added, etc., as for (1) | 1.0 |
| 3. Control, no charcoal | 1.3 |

SUMMARY.

1. Charcoal removes trypsin from solution. The amount removed depends on the order in which the solutions are mixed. The reaction is not reversible and is almost independent of the pH of the solution.

2. Charcoal which has been previously treated with gelatin does not remove trypsin from solution.

3. The reaction is not analogous either to the reaction between trypsin and the inhibiting substance of serum or to the reaction between solid protein and either pepsin or trypsin.

# STRUCTURAL UNITS OF STARCH DETERMINED BY X-RAY CRYSTAL STRUCTURE METHOD.

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Among the earliest investigators of starch the idea prevailed that these minute, transparent, spherical grains were built of uniform structural units, the nature, size, shape, and arrangement of which have been the source of a number of theories and of much speculation. Raspail's work[1] in 1825 led him to think that the grains were not crystallization products. Münter[2] on the other hand decided that the grains must be crystals. Nägeli conceived the grain to be made up of concentrically and radially arranged particles which he called at one time[3] molecules, and later[4] micellæ, and in one place he speaks of these small particles as consisting of several thousand molecules. In 1863 Kabsch[5] considered starch grains to have a very definite arrangement of molecules, although not sufficiently uniform to have optical axes ascribed to them. Schimper[6] decided that they consisted of radially arranged crystal fibers. In 1895 Meyer[7] completed this idea of a spherocrystal and conceived the grains to be built of radially arranged needle-like branching crystals, which became more and more branched the more nearly they approached the surface of the grain. These crystals he called trichites. The vast amount of work that has been done since then has neither confirmed nor disproved the theory of the trichite or micellar structure, and of the later articles on starch some favor and some oppose this view.

[1] Raspail, F. V., *Annal. sc. natur.*, 1825, vi, series 1, 387.
[2] Münter, J., *Bot. Ztg.*, 1845, iii, 194.
[3] Nägeli, C., Die Stärkekörner, Zürich, 1858.
[4] Nägeli, C., *Bot. Ztg.*, 1881, xxxix, 637.
[5] Kabsch, W., *Z. anal. Chem.*, 1863, ii, 216.
[6] Schimper, A. F. W., *Bot. Ztg.*, 1881, xxxix, 223.
[7] Meyer, A., Untersuchungen über die Stärkekörner, Jena, 1895.

Probably all has been done with the microscope and the ultramicroscope that can be done, and no structural unit has been found. If one is to be discovered it must probably be with some new tool, and during the past few years such a new tool has made its appearance, namely the x-ray. It has been used to investigate the building units of crystals, and with it a great deal of work has been done in determining the size and arrangement of these structural units in many inorganic crystals. These units prove to be the atoms of which the crystals are composed. For example, the cubical crystal of common table salt consists of sodium and chlorine atoms arranged alternately in three dimensions. The position with relation to each other and the distance from center to center of these atoms have been very accurately determined.[8] About fifty compounds and a considerable number of metal elements have been investigated in this way, among them the diamond, graphite, carbonates, sulfides, oxides, etc. Very few organic compounds, however, have been studied.

With these investigations in mind it seemed fairly plausible that the structure of the starch grain might be determined, especially since the grains behave towards light as though they were built up in some regular way, and since x-rays are of the nature of light.

The x-ray method of determining the atomic structure of crystals is described in considerable detail in a number of places in the literature,[8,9,10] so only a very brief resumé will be given here. For proofs and evidence concerning the various steps the reader is referred to the citations just mentioned.

The method consists in measuring the angle at which x-rays are reflected from a crystal, and computing from that measurement the distance between the planes of atoms which have caused the reflection. When an x-ray wave is incident on the face of a crystal it passes through the surface layer of atoms and through many thousand

[8] Bragg, W. H., and Bragg, W. L., X-rays and crystal structure, New York and London, 1918.

[9] Hull, A. W., *Phys. Rev.*, 1917, x, series 2, 661; 1921, xvii, series 2, 571. Debye, P., and Scherrer, P., *Physik. Z.*, 1916, xvii, 277. Bain, E. C., *Chem. and Metallurg. Eng.*, 1921, xxv, 657.

[10] Sponsler, O. L., *Am. J. Bot.*, 1922, ix, 471.

layers beneath. Only a minute part of the wave is reflected from each plane. There are several millions of these planes or layers uniformly spaced in each millimeter of depth in a crystal. The distance between the layers and also between the atoms in the layer, varies with the kind of crystal from one to several Ångström units. An Ångström unit is $\frac{1}{10{,}000{,}000}$ millimeter. When the distance between the planes is such that a crest of the minute part of the wave which is reflected from one plane coincides with a crest from that of the next plane, then they will reinforce, and the sum of many thousand such reflections will carry sufficient energy to register visibly on a photographic plate. Thus x-ray reflection depends upon reinforcement from a depth of many planes. The crests of the waves will coincide only when there is a certain relation between the glancing angle, the wave length of the x-rays used, and the distance between the planes. This relationship is expressed by the formula

$$\lambda = 2d \sin \theta$$

in which $\lambda$ is the wave length, $d$ the distance between planes, and $\theta$ the glancing angle. If the glancing angle varies by only one part in several thousand the tendency is to annul instead of reinforce, and consequently, reflection can occur only at a very definite angle for a given set of planes and a given wave length.

When a narrow beam of x-rays, consisting primarily of one wave length of known value, is allowed to pass through a small mass of powdered crystals, some of the crystal particles will reflect at one angle, some at another, still others at a third angle, and so on, according to the relation mentioned above between the wave length, the glancing angle, and the distance between planes in the crystal particles. When these reflections are recorded on a photographic film, they will appear as dense lines with a width corresponding to the slit through which the beam of x-rays is allowed to pass before reaching the powder. The arc of the reflecting or glancing angle can be measured in millimeters directly on the film, and since a known wave length is used, the distance between planes can be readily computed. For a more detailed and simpler explanation, as well as

for diagrams and descriptions of the apparatus used in this work, the reader is referred to my paper in the *American Journal of Botany*.[10]

Many demonstrations have been made by a number of investigators to show that the dense lines, as in Fig. 1, are produced by the reflection of x-rays from planes of atoms. These demonstrations consist of two parts. First, that the lines on the photograph are produced by reflecting points arranged in planes; and second, that these reflecting points are atoms. The proof of the first part consists in obtaining an agreement between the values of $d$ (distance between planes) from measurements of the photograph, and the values obtained from a mathematical consideration of the type of crystal used. The proof of the second part consists in obtaining an agreement between the volume occupied by a reflecting point as computed from the photograph and the volume of the atom or molecule as obtained by the use of the molecular weight, the density, and Avogadro's number. It may be worth while to illustrate these two points briefly, since similar methods are used later in determining the structural units of starch.

Fig. 1 is reproduced from an x-ray photograph of the lines reflected by the crystal particles of powdered sodium chloride. A rhodium anticathode was used, and the rays were filtered through a ruthenium screen so that the beam was practically monochromatic. The blank space in the middle of the photograph is the shadow of a lead shield used to absorb that part of the main beam which was not diverted when passing through the powder. The right and left hand sides are mirror images, and the distances between lines 1 and 1, 2 and 2, 3 and 3, etc., measure the arc of $4\Theta$ in each case, where $\Theta$ is the glancing angle. By using these measurements and the rhodium $K\alpha$ wave-length 0.614 Å.u., the distance between the planes of reflecting points was computed from the fundamental formula, $\lambda = 2d \sin \Theta$. The results show that line 1 was reflected from planes which were 2.81 Å.u. apart; line 2, by planes 1.99 Å.u. apart and so on as recorded in Table I.

A striking agreement is obtained when these figures are compared with those which were computed from a cubical space lattice such as Bravais' old theory proposed for the atoms in a crystal. Knowing that sodium chloride forms cubical crystals, we may think of a

unit cube as having an atom at each corner and of the large crystal as being built up of many of these unit cubes with the atoms shared by the adjacent cubes. The atoms then would form a space lattice in which they would occur in many sets of parallel planes. The planes coinciding with the faces of the cubes would be farthest apart, and the distances between the diagonal planes would bear definite relations to this widest spacing. When the sides of the cube are

TABLE I.

*Sodium Chloride.*

| Line No. | Distance between planes. |
|---|---|
| | Å.u. |
| 1 | 2.81 |
| 2 | 1.99 |
| 3 | 1.62 |
| 4 | 1.41 |
| 5 | 1.26 |
| 6 | 1.14 |

TABLE II.

*Sodium Chloride.*

| Line No. | Distance computed from angle and wave-length. | Computed from cubic lattice based on 2.81 Å.u. | Ratio of planes in a cubic lattice. |
|---|---|---|---|
| | Å.u. | Å.u. | |
| 1 | 2.81 | 2.81 | 1.000 |
| 2 | 1.99 | 1.99 | 0.707 |
| 3 | 1.62 | 1.62 | 0.577 |
| 4 | 1.41 | 1.41 | 0.500 |
| 5 | 1.25 | 1.25 | 0.447 |
| 6 | 1.14 | 1.14 | 0.408 |

1.00 unit apart the various sets of diagonal planes are spaced 0.707 unit, 0.577, 0.500, etc. Now if we accept line 1 as being reflected by the planes which have the widest spacing, then if a cubical arrangement exists, line 2 would be reflected by planes which are spaced $0.707 \times 2.81$; line 3, $0.577 \times 2.81$, etc. Table II gives the ratio of spacings of planes in a cubic lattice and the distance between planes based on 2.81 Å.u. as the possible distance between cube faces. Table I is incorporated in it for comparison.

Such close agreement between these two sets of figures necessarily leads one to conclude that the "reflecting points" must be arranged in a cubic space lattice.

The second part of the demonstration, showing that the reflecting points are atoms, is given briefly in the following paragraphs.

It is a well established fact that the molecular weight of any compound in grams contains a definite number of molecules; the accepted figure is $6.062 \times 10^{23}$. This is called Avogadro's number. Sodium chloride contains that number of molecules for each 58.5 gm. of the salt. The volume of 58.5 gm. of NaCl is 26.95 cc., since its specific gravity is 2.17.

$$\frac{58.5}{2.17} = 26.95$$

That volume then contains $6.062 \times 10^{23}$ molecules, and the volume of 1 molecule of sodium chloride would be $\frac{26.95}{6.062 \times 10^{23}}$ or $44.4 \times 10^{-24}$, or 44.4 cu. Å.u.

The photograph shows that in the unit cube the faces are spaced 2.81 Å.u. apart. Each point, then, might be considered as having a volume of $2.81^3$ cu. Å.u., and if we assume each to be an atom, then the volume of two of them should be the volume of a molecule of sodium chloride.

$$2.81^3 \times 2 = 44.4 \text{ cu. Å. u.}$$

Agreement as close as that both in spacing and in volume makes it difficult to believe anything else than that the reflections are due to atoms arranged in a cubic space lattice.

### *Interpretation of the Starch Spectrum.*

In an earlier article[10] it was shown that an x-ray spectrum can be obtained from a mass of starch grains somewhat similar to that obtained from the crystal powder of sodium chloride. In later work, which is described in the present paper, the nature and the size of the reflecting unit have been determined and a definite structure has been ascribed to the starch grain. The demonstration of these conclusions following somewhat the same lines of reasoning as just used for sodium chloride, involves the several steps which are now to be presented.

*Nature of Reflected Lines.*

When compared with a line photograph of a common crystalline substance such as sodium chloride, the negatives from starch exhibit several striking features (Figs. 1 and 2). The background is very dense and the lines are only slightly denser, consequently the definition of the lines is poor; they seem to blend in with the background instead of being clear cut, and wherever they occur close

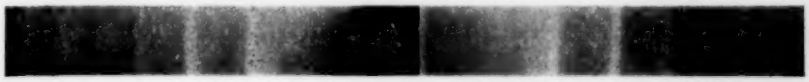


FIG. 1. Sodium chloride.

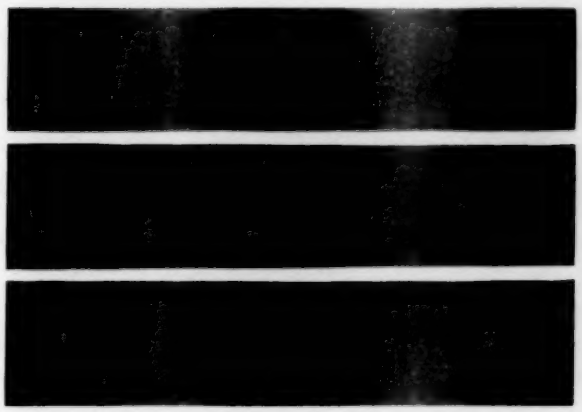


FIG. 2. Top section, potato starch; middle section, cassava starch; bottom section, corn starch.

together they often overlap so much that two lines make a broad band in which they are scarcely separable. These features tend to make measurements, both of distance and of comparative density between lines, very difficult. They also militate against a satisfactory reproduction of the photographs by means of half-tones.

About a dozen negatives were made from three different kinds of starch—potato, corn, and cassava. On all of them two or three of the lines are noticeably prominent and can be measured with a

fair degree of accuracy, several more are visible only when carefully studied in properly adjusted light, and still two or three more can be detected only with a photometer. The first two lines are sometimes blended into a single broad band, but on several negatives they appear clearly separated and there they can be readily measured. Line 4 (Table III), the third prominent line from the center of the negative is by far the most sharply defined and has yielded consistent measurements on all of the ten or eleven negatives. Of the other lines, 6, 7, 8, 9, 11, and 12 are distinct enough on a half dozen or more negatives to be fairly consistently measured. The actual existence of some of the lines was often questioned on account of their faintness, but several months after this paper was written an opportunity was presented for having an energy curve made on a recording micro photometer,[11] and the position of the lines as shown by the curve agreed so well with the measurements of the negatives that further doubt of their existence was dispelled. The curves, in addition to verifying the visual location of the lines, brought out two lines, 3 and 5, which had been predicted but had not been distinguished by the unaided eye.

The fact that lines are present on the negatives indicates the existence of reflecting points arranged in some regular way forming a space lattice. In a space lattice the units form many different sets of parallel planes. Each set, having its own particular value of $d$, the distance between planes, produces one of the lines on the photographic film. The value of that $d$ may be determined by using the formula $\lambda = 2d \sin \Theta$, in which the value used for $\Theta$ is obtained from measurements of the lines on the negative. In this way the value of $d$ for every line found on the negatives was obtained and listed in Table III, Columns 2 and 3. The value of $\lambda$ used in these computations was 0.614 Å.u., the wave length of the $K\alpha$ line of rhodium. The figures, then, in the table represent the distances between the parallel planes of the various sets for an unknown space lattice.

[11] Through the courtesy and kind assistance of Mr. Edison Pettit of the Mount Wilson Observatory at Pasadena, California, the energy curves referred to were made on the registering micro photometer described in a paper by Pettit and Nicholson (Pettit, E., and Nicholson, S. B., *J. Optical Soc. Am. and Rev. Scient. Instruments*, 1923, vii, 187).

The problem now is to determine the type of this unknown space lattice and the dimensions of its elementary cell. The solution was influenced by the one well established fact concerning the formation of the starch grain in the plant cell; that is, that the starch substance is deposited on the grain at the interface between the grain and the protoplasm.[12] Since the grain assumes a more or less sperical form it seems probable that the particles are deposited at equally spaced points on the surface of the grain. This would make two dimensions of the lattice equal and the value would be the largest obtain-

TABLE III.

*Starch.*

| Line No. | *d* from photometer curves. | *d* from visual measurement. | *d* calculated from lattice, 5.94 × 5.94 × 5.05. | Indices of planes. |
|---|---|---|---|---|
| | Å.u. | Å.u. | Å.u. | |
| 1 | 5.94 | 5.94 | 5.94 | 001, 100 |
| 2 | 5.05 | 5.05 | 5.05 | 010 |
| 3 | 4.25 | —— | 4.17 | 101 |
| 4 | 3.87 | 3.87 | 3.85 | 110, 011 |
| 5 | 3.28 | —— | 3.20 | 111 |
| 6 | 2.92 | 2.98 | 2.97 | 001, 100 2nd order |
| 7 | 2.61 | 2.65 | 2.64 | 201, 102 |
| 8 | 2.50 | 2.53 | 2.53 | 010 2nd order. |
| 9 | —— | —— | 2.55 | 210, 012 |
| 10 | —— | 2.33 | 2.30 | 120, 021 |
| 11 | 2.05 | 2.10 | 2.09 | 101 2nd order. |
| 12 | 1.90 | 1.94 | 1.93 | 110, 011 2nd order. |

able from the negatives; that is, 5.94 Å.u. The third dimension would be likely to be the next larger spacing, 5.05 Å.u. and the elementary cell of the lattice then would have the dimensions, 5.94 × 5.94 × 5.05. If this cell is a rectangular figure, a square prism, then it would have for its various planes the values of *d* as given in Column 4 of Table III. The indices of the planes are given in Column 5 and the position of the planes in the cell is shown in Fig. 3.

[12] Salter, J. H., *Jahrb. wissensch. Bot.*, 1898, xxxii, 164. Crüger, H., *Bot. Ztg.*, 1854, xii, 41. Schimper, A. F. W., *Bot. Ztg.*, 1880, xxxviii, 884. Strasburger, E., Über den Bau und das Wachstum der Zellhäute, Jena, 1882, 154–159.

The agreement between these values and those obtained from the measurements of the negatives is sufficiently close to justify our acceptance of the shape and the size of the elementary cell as assumed above, and we may now say that the lines on the negatives indicate that planes of reflecting units are spaced 5.94 Å.u. apart, and that other planes at right angles to those are spaced 5.05 Å.u apart, forming elementary cells of the size mentioned in the last paragraph. The lattice would look somewhat like Fig. 4.

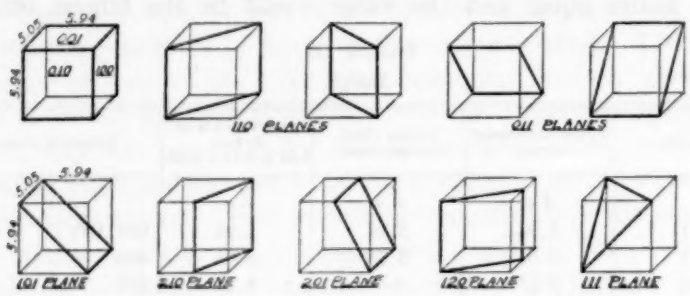



FIG. 3. Position of the more important planes in the elementary cell.

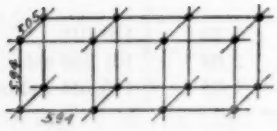



FIG. 4. Elementary cells from the lattice of the starch grain.

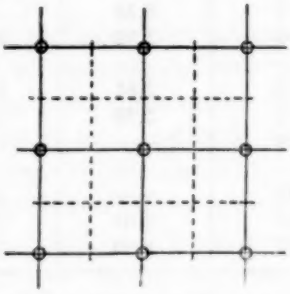


FIG. 5. Elementary cells in two positions.

*Size and Nature of the Reflecting Unit.*

The atoms which cause the reflection from starch grains are those of carbon and of oxygen. The former are 1.50 Å.u. in diameter and the latter, 1.30 Å.u. The reflection from hydrogen is too weak to make any appreciable difference in this work. It seems very probable that the units which form the lattice are not single atoms, for a structure with so great a distance as five or six

Ångström units between atoms, which are no larger than those of carbon and oxygen, would take the form of some other state of matter than that of a solid with the density of starch. It follows then that there must be a *group* arrangement of the atoms around each corner of the elementary cell. An arrangement of this nature is not unusual. Very convincing evidence has been brought forward to show that the benzene ring acts as a unit in crystals of anthracene, naphthalene, and their compounds,[13] that the $CO_3$ group retains its identity in crystals of carbonates, and that 3 molecules of silicon dioxide are associated with each point in the lattice of quartz crystals.[14]

The group of atoms in starch, of which one would naturally think first, is that indicated by the empirical formula, $C_6H_{10}O_5$. The volume of that group as computed from the density of starch, 1.50, the sum of the atomic weights, 162, and Avogadro's number, $6.062 \times 10^{23}$, is 178.2 cubic Ångström units.

$$\frac{162}{1.50} \times \frac{1}{6.062 \times 10^{23}} = 178.2 \times 10^{-24} \text{ or } 178.2 \text{ cu. Å. u.}$$

If that is the group which is associated with the corners of the cells, then its volume, 178.2 cu. Å.u. should be equal to the volume of the elementary cell. Although we have thought of the groups as being identified with the corners of the cells, that is the points of the lattice, the imaginary boundary lines may be shifted so that a group will be seen to occupy a space equal to that of the cell. In Fig. 5 the dotted lines indicate the positions of the new cells after the shift has been made. The volume of this cell is almost exactly that of the group, 178.2 cu. Å.u.

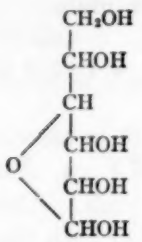
$$5.94 \times 5.94 \times 5.05 = 178.2$$

This agreement between the volumes of the elementary cell and the group of atoms is perhaps closer than is consistent with the degree of accuracy of the measurements; on the other hand, it is not likely that it is merely coincidental. It seems to me that we may accept the figures as indicating the probable structure and that we

[13] Bragg, W. H., *Proc. Phys. Soc. London*, 1921, xxxiv, 33.
[14] Bragg, W. H., and Bragg, W. L.,[8] pp. 117 and 164.

may consider the starch grain as built up of *groups* of atoms, each group being associated with each point in the lattice, that is a group acting as a unit of the lattice; and further, that each group consists of 6 carbon, 5 oxygen, and 10 hydrogen atoms.

It is highly probable that each group ($C_6H_{10}O_5$) has a definite configuration, and further, that all of the groups, at least a large percentage of them, are oriented in the same way with respect to the surface of the grain. These assumptions are based on the dark cross-effect with polarized light, a common phenomenon produced by starch grains. What the configuration of that group is we have no means of knowing at the present time, but we may assume a form for illustration which has a slight basis in the chemistry of starch. The end-product of many starch reactions is glucose ($C_6H_{12}O_6$). The structural formula generally accepted for glucose shows the 3rd and 6th carbon atoms linked together through an oxygen atom.




In the starch group the 6 carbon atoms are attached, without doubt, in the same way and it is not improbable that the 3rd and 6th carbon atoms are linked also through an oxygen atom. On that assumption a model of the starch group was made to scale.

Fig. 6 gives two views of the model. The dark balls represent the carbon atoms, the light ones the oxygen atoms, and the small loops indicate the position of the hydrogen atoms. So many arrangements of the atoms and so many orientations of the whole group inside of the elementary cell are possible that no specific conclusions can be made, but nevertheless certain rather convincing impressions are left with one after a short study of this hypothetical structure.

The size of the structure corresponds to the size of the elementary cell, the boundary lines of which are represented in the model by the rectangular wire cage. The group fits in the cell loosely enough

to suggest the low specific gravity of starch and at the same time extends far enough towards each face of the cell to make the rather weak cohesion, which is characteristic of the starch grain, seem possible.

In Fig. 6 the diagrams below the pictures of the model are intended to help make clear the kind of reflection which occurs when a group of atoms acts as a unit of the lattice. Parallel to each face of the

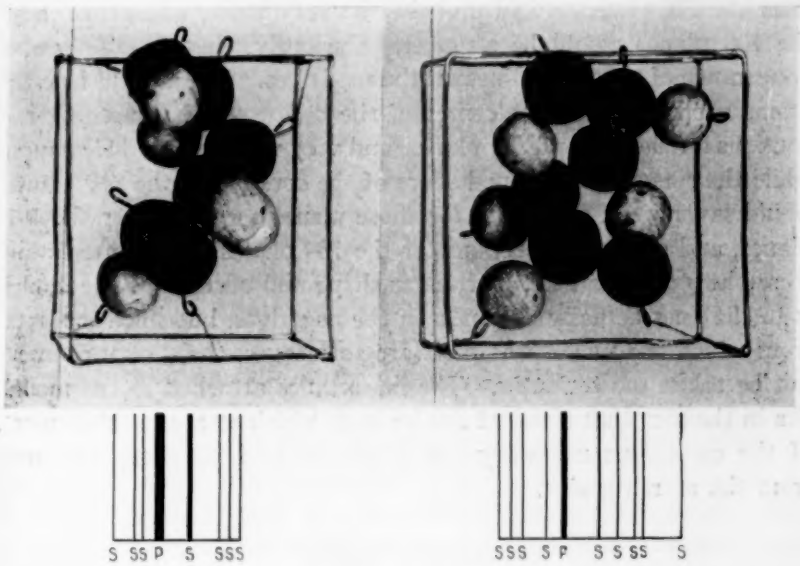



FIG. 6. Model of starch group. Principal and secondary planes indicated by lines below.

cell there are several possible planes which contain atoms of the unit group. In the 100 view the plane marked *p* in the diagram contains 4 atoms, the others marked *s* contain only 1 each, or in one case, 2 atoms. In the 010 view the principal plane, *p*, has 2 atoms in it and each of the secondary planes, *s*, has only 1. The reflection which produces a line on the negative comes from the principal plane, but its reflecting power may be partially annulled by waves from the secondary planes. The tendency of secondary planes in

general is to annul each other, depending upon the spacing and the number of atoms occurring respectively in them. Thus the reflecting power of a particular set of planes depends not only on the number of atoms in a unit area of the principal plane, but also upon the conditions of spacing and number of atoms occurring in the secondary planes. It is evident from this and the two views of the model that for the same group of atoms the reflection from one set of planes will not necessarily be as strong as that from another set. The reflection from the 010 planes would probably be very weak, while that from the 100 planes would be stronger. Curiously, that is exactly the appearance of those two lines on the negatives. The core of heavier atoms occurring near the center of the cell would favor strong reflections for the 110 and 011 planes, and very weak for the 111 planes, while the somewhat flattened effect of the core facing the 010 planes would favor weak reflections for those planes as well as for the 101 planes, and stronger reflections for the 100 planes. It is interesting to see how favorable this particular shape and position of the model is to the lines actually obtained on the negatives, but since the data from which the model was built are so meager, the structure must not be taken too seriously. The importance attached to the model lies in the fact that a model can be built which seems to fulfill most of the conditions necessary for reflections such as those obtained from the starch grains.

### *Concentric Layers.*

Up to this time the data have been used as though starch were without question a crystalline substance, while as a matter of fact starch does not have the appearance nor the properties of common crystals. The grains are spherical bodies which have grown by additions at the interface between the grain and the plastid, that is they are built up of concentric layers; they have no ordinary cleavage planes; and further, the polarized light phenomenon is not that of ordinary crystals. These statements immediately raise several questions. How could spherical grains produce line photographs similar to those of ordinary crystals? How could a lattice such as that ascribed to starch occur in a sphere?

These questions may be answered best perhaps by comparing the photographs from starch with the results of an assumption that the grains are built up of concentric layers, each layer having the thickness of the $C_6H_{10}O_5$ group which, from the lattice worked out above, would be 5.05 Å.u. thick, and the groups in each layer located 5.94 Å.u. apart. In other words the layers would be sheets of elementary cells, one cell thick. Of course the inner layers would have to contain a smaller number of cells than the outer ones, and in order to retain the rectangular lattice there would have to be slight readjustments. The result of these readjustments may be the so called lamellæ of starch grains.

To gain a better conception of this arrangement one may imagine the elementary cells to be the size of common building bricks, and then build up a set of concentric circles to represent a cross-section of a starch grain. Now in the average starch grain there are about 20,000 elementary cells along one diameter. (The grains are about 100,000 Å.u. in diameter.) So the concentric circles of bricks would be built out until the diameter is 20,000 bricks. This would make the outside circle about $1\frac{1}{2}$ miles in diameter.

One can see that the rearrangement of elementary cells in the inner circles, in order to keep a rectangular lattice, would be comparatively insignificant; and that large regions would closely approach the parallel layers of a rectangular lattice, such as that of ordinary crystals. It seems then not improbable that starch grains built proportionally in a somewhat similar way would reflect x-rays from certain regions of the grain. Each spherical grain, unlike the particles of a crystal powder, however, would reflect from a great many regions. From this point of view it is interesting to examine into the possibilities of reflection from a powder composed of such spherical grains.

In Fig. 7 suppose the large circle to be a cross-section of one of these spherical grains placed in the path of a beam of x-rays. The regions indicated by the letters are drawn to show four elementary cells. The reflecting planes are made somewhat heavier than the other lines. Of course the cells are enormously out of proportion to the diameter of the circle.

The strongest reflection would be that from the 3.85 planes since every grain would reflect from at least the four regions C, C, C, C, and many of them which happen to be revolved into certain positions would reflect from four additional regions, one above and one below each B. On all of the negatives this line is actually the most prominent line.

Every grain would reflect also from the 5.05 planes from two regions, B, B. This line too should be a strong line, about as dense

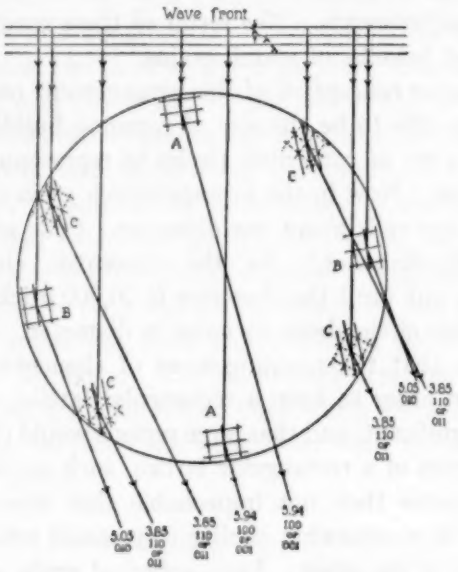



FIG. 7. X-ray reflection from a starch grain.

as the 3.85 line. The negatives actually show these two lines to be about equal in intensity.

The 5.94 planes would be in the proper position to reflect in only a comparatively few grains and on that account should give a much weaker line than the 5.05 planes; but when in that position, the reflecting region would completely encircle the grain passing through A, A, and consequently the density of the reflected line should approach that of the 5.05 line. This is in fairly satisfactory agreement with the negatives.

The 4.17 planes would reflect in similar positions to those of the 5.94 planes and should be, as in ordinary crystals, somewhat weaker. On the negatives the lines are actually weaker than one is lead to expect from the diagram.

The 3.20 planes, which would reflect from four or eight positions just as it was shown the 3.85 planes would do, actually produce much weaker lines than would be anticipated.

In making these comparisons no consideration was given to the probable fact that the principal planes of the different positions are not equal in reflecting power, nor to the possible effect of the secondary planes in reducing the reflection. Both the position of the secondary planes and the number of atoms in them are very important factors in determining the density of the lines, but there are absolutely no data so far obtained which could be used in this connection. On the whole the agreement between the negatives and the expectations from the diagram is fairly satisfactory.

There is another factor which may tend to make the lines less dense and so diminish the difference in intensity between the lines and the background. The 21 atoms of the starch group, while they occupy a space equal to 178 cubic Ångström units, undoubtedly do not completely fill that space. On the assumption that the carbon and oxygen atoms are the same size in starch that they appear to be in many crystals, that is 1.5 Å.u. and 1.3 Å.u. in diameter respectively, and guessing that the hydrogen atoms are about 1 Å.u. in diameter, the total space which these atoms would fill is less than 50 cubic Ångström units. The unoccupied space in the elementary cell would be four or five times as much as that actually filled with atoms. The group then is loosely constructed and is likely to be strongly influenced by temperature. In fact, an increase of about 150°C. over room temperature produces profound changes in dry starch, and in water at about 60°C. the grains undergo a kind of disintegration. Based on that, one might expect considerable thermal agitation in dry grains even at room temperature. It has been shown[15] that there is a very decided reduction in intensity of reflection from sodium chloride when raised

[15] Bragg, W. H., *Phil. Mag.*, 1914, xxvii, 881.

in temperature about half way up to its melting point. The experimental proof of the thermal effect in the case of starch seems comparatively simple but has not yet been attempted.

Still another factor which should be mentioned in connection with the density of the reflected lines is one embodied in the spherical shape of the grain. While certain large regions of the grain act as ordinary crystal particles, there are other regions which just miss the exact angle for reflection and in which there are not enough planes to annul completely the reflection. This would produce a broadening of the reflected lines and increase the density of the background by blending into it.

## SUMMARY.

A few brief statements summarizing the foregoing conclusions may make a picture of the structure of the starch grain somewhat clearer.

1. The presence of lines on the negatives indicates a regular arrangement of the planes of atoms.

2. The lines are in close agreement with lines which would be produced by a lattice of the tetragonal system, the elementary cell of which is a square prism with the dimensions $5.94 \times 5.94 \times 5.05$ Å.u.

3. The unit of the lattice occupies a space equal to the volume of the starch group, $C_6H_{10}O_5$.

4. The large number of atoms in the unit makes it highly probable that principal planes and secondary planes of atoms occur for every reflecting position.

5. The effect of the secondary upon the principal planes may readily account for the differences in the density of the lines produced on the negatives.

6. From theoretical considerations, reflections, such as those obtained, would occur if starch grains were built up of concentric layers of units.

7. Two other factors which might affect the density of the lines are thermal agitation and the curvature of the concentric layers.

8. A model of the starch group was constructed to scale based on the accepted sizes of the atoms involved and upon rather meager chemical evidence. The model apparently fulfills the requirements necessary to produce reflections such as were obtained.

9. The model fits the elementary cell loosely enough to suggest a low density and to allow for considerable thermal movement. At the same time, parts of it approach the faces of the cell closely enough to make cohesion seem possible.

10. The model makes clearer the basis for the assumption that reflection from certain positions would be stronger than from others.

If the interpretation of the data is correct and if the assumptions made are sound, then the starch grain is built up of units arranged in concentric layers, and the units are groups of atoms, each containing 6 carbon, 10 hydrogen, and 5 oxygen atoms. Such a structure is certainly not an amorphous structure, and on the other hand it is not crystalline in the common sense of the term. Parts of the grain, it is true, act as crystals in that for certain distances the layers of units are in planes, but taken as a whole the layers are curved.

As to the validity of the conclusions, those pertaining to the type of lattice and to the size of the unit may be accepted as sound in our present knowledge of x-rays and crystal structure; those, however, pertaining to the nature and the spherical arrangement of the units, while they seem convincing, need the support of further investigation into the various structures deposited by living protoplasm.

In conclusion, the assumption that the units form a sort of spherical space lattice, gives a picture of the starch grain which leads us to ponder over the nature of the activity in protoplasm when it is depositing solid substances. Starch, cellulose, and pectic bodies are about the only solid deposits made directly by the living substance of plants, and all three have the same proportional formula, $C_6H_{10}O_5$. Investigations, as yet incomplete, indicate that cellulose also consists of a regular arrangement of $C_6H_{10}O_5$ groups, each acting as a unit, but the spacing ($6.14 \times 6.14 \times 5.55$) is slightly different from that of starch. Pectin has not been studied. Protoplasm may be thought of as being composed of molecules of many different sizes, polypeptides, or even proteins forming the larger, and amino-acids the smaller, if water and electrolytes are ignored. The smaller molecules, such as those of the amino-acid, leucine, are approximately equal in size to the $C_6H_{10}O_5$ group of starch. That being the case, what can be the state of affairs at the interface when the starch particles are being deposited? Is it probable that protoplasm is

homogeneous to the extent of being able to deposit these particles at 6 Å.u. intervals?

From quite another view-point a clear picture of the units of structure and their arrangement in cellulose should give a new point of attack on the many problems connected with osmosis. And from still a different view-point, it might lead perhaps, to a solution of problems connected with swelling.

Another line of thought is suggested by the uniformity of the groups in the starch grain. Since the $C_6H_{10}O_5$ group occurs as an individual unit, one is inclined to suspect that it is really the molecule. Generally the starch molecule is considered to be very large, to be composed of several dozens of such groups, and to have a molecular weight of 7,000 or much more. No one figure, however, seems satisfactory to the different authorities. There is already at hand considerable evidence which will be assembled in a later paper favoring the single group, $C_6H_{10}O_5$, as the molecule.

Finally, problems in polarized light may receive more satisfactory explanations through a clearer notion of the molecular structure of the carbohydrates.

It is a pleasure to mention here my appreciation of the suggestions and of the help kindly given me by Dr. D. L. Webster and Dr. George J. Peirce at Stanford University.

# THE EFFECT OF GESTATION ON THE RATE OF DECLINE OF MILK SECRETION WITH THE ADVANCE OF THE PERIOD OF LACTATION.

By SAMUEL BRODY, ARTHUR C. RAGSDALE, and CHARLES W. TURNER.

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(Received for publication, May 12, 1923.)

In the last communication[1] it was shown that from the 2nd to the 12th month of lactation, the course of decline of milk secretion of the dairy cow with the advance of the period of lactation is very nearly exponential; that is, each month's milk production is nearly a constant percentage of the production of the preceding month. In two of the groups of animals—the Guernsey and Jersey cows consisting of a large number of animals on official test—the fit of the exponential equation to the data was quite satisfactory except for a slight deviation for the last 1 or 2 months. In the case of the Holstein and Scrub cows not on official test, and where the number of animals was limited, the observed values of the last months of lactation tended to fall considerably below the values computed from the exponential equation. It was suggested that the condition of advanced pregnancy during the last months may be the cause of this discrepancy. It is, in fact, commonly believed by experienced dairymen that gestation is an important factor in bringing about a decreased milk flow, and for this reason breeding is usually delayed in cows on official test. We have investigated gestation as a possible factor in bringing about the discrepancy between the observed and calculated values for the decline of milk secretion with the advance of the period of lactation, and it is the purpose of this communication to report the results.

1. One method of investigating this problem consisted in dividing milk records into two groups; a farrow group, in which the cows were not bred during the year under observation, and a pregnant group, in which the animals were bred during the 3rd and 4th months after calving, which brings the last month of gestation to the 12th month

[1] Brody, S., Ragsdale, A. C., and Turner, C. W., *J. Gen. Physiol.*, 1922–23, v, 441.

of lactation (the period of gestation in the cow being about 9⅓ months). The averages for these two groups of cows are presented in Table I and Fig. 1.

TABLE I.

*The Relative Decline of Milk Secretion with the Advance of the Period of Lactation of Farrow and Gestating Cows (Guernsey Breed).*

| Month of lactation. | Farrow cows. | | | Gestating cows. | |
|---|---|---|---|---|---|
| | No. of animals. | Yield per month. | | No. of animals. | Yield per month. |
| | | Observed. | Calculated.* | | Observed. |
| | | *lbs.* | *lbs.* | | *lbs.* |
| 2 | 920 | 1,052 | 1,049 | 373 | 1,052 |
| 3 | 912 | 998 | 994 | 374 | 983 |
| 4 | 923 | 938 | 942 | 373 | 911 |
| 5 | 914 | 879 | 874 | 372 | 862 |
| 6 | 917 | 833 | 846 | 372 | 819 |
| 7 | 912 | 792 | 802 | 374 | 776 |
| 8 | 904 | 752 | 760 | 371 | 728 |
| 9 | 905 | 715 | 720 | 369 | 679 |
| 10 | 906 | 676 | 682 | 369 | 618 |
| 11 | 852 | 650 | 641 | 358 | 538 |
| 12 | 653 | 617 | 613 | 283 | 469 |

* Calculated from the equation $M_t = 1167.2\ e^{-0.0537\,t}$ where $M_t$ is the milk production during any month of the lactation period, $t$.

From these data it is evident that the decline in milk secretion of the farrow group of animals follows almost exactly the course of the exponential equation[2]

$$M_t = M_o e^{-kt}$$

[2] $M_t$ is the milk produced during any month of the lactation period, $t$; $M_o$ and $k$ are constants. It may be remarked incidentally that the total amount of milk a farrow cow is capable of producing due to one pregnancy approaches $\frac{M_o}{k}$ as a limit since $\int_o^\infty M_o\, e^{-kt}\, dt = \frac{M_o}{k}$. For the Guernsey cow under consideration, $\frac{M_o}{k} = \frac{1167.2}{0.0537} = 21{,}735$ lbs. of milk. This is only a little over twice the amount of milk secreted during the 1st year of lactation. Theoretically, the milk produced during the 1st year is 10,050 lbs.; during the 2nd, 5,276; during the 3rd, 2,770; during the 4th, 1,453; and during the 5th, 763; each year's production is 52.48 per cent of the preceding year's production, or each month's production is 94.77 per cent of the preceding month's production.

thus substantiating the suggestion of the preceding communication[1] that the decline of milk secretion with the advance of the period of lactation is limited by a chemical reaction when other factors, such as pregnancy, are eliminated.

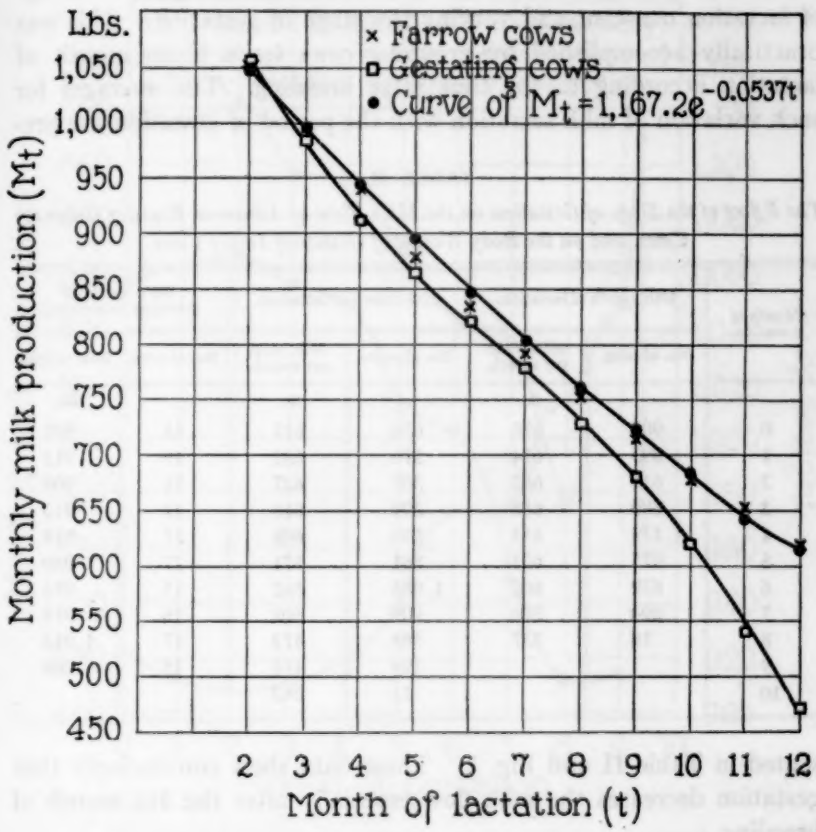


FIG. 1. The relative decline of milk secretion with the advance of the stage of lactation of farrow and gestating Advanced Registry Guernsey cows.

That pregnancy materially affects the course of decline of milk secretion with the advance of the period of lactation, especially after the 5th month of breeding, is evident enough from the decline curve of the pregnant groups of cows. Pregnancy then, no doubt, was the

chief cause of this discrepancy between the observed and calculated values for the decline of milk secretion with the advance of the period of lactation.

2. Another method of investigating this problem concerning the effect of pregnancy on milk secretion consisted in keeping the stage of lactation constant and varying the stage of gestation. This was practically accomplished by grouping cows for a given month of lactation according to the time after breeding. The averages for such variation of milk secretion with the period of gestation are pre-

TABLE II.

*The Effect of the Stage of Gestation on the Milk Flow of Advanced Registry Guernsey Cows, and on the Body Weight of Gestating Jersey Cows.*

| Month of gestation. | 10th month of lactation. | | 12th month of lactation. | | Body weight of gestating Jersey cows. | |
|---|---|---|---|---|---|---|
| | No. of cows. | Milk yield per month. | No. of cows. | Milk yield per month. | No. of cows. | Body weight. |
| | | *lbs.* | | *lbs.* | | *lbs.* |
| 0 | 907 | 676 | 654 | 617 | 15 | 921 |
| 1 | 393 | 674 | 276 | 622 | 16 | 912 |
| 2 | 616 | 682 | 307 | 627 | 16 | 908 |
| 3 | 890 | 658 | 399 | 616 | 17 | 910 |
| 4 | 1,133 | 655 | 593 | 608 | 17 | 919 |
| 5 | 975 | 627 | 861 | 571 | 17 | 940 |
| 6 | 659 | 602 | 1,085 | 562 | 15 | 973 |
| 7 | 294 | 596 | 909 | 508 | 16 | 975 |
| 8 | 18 | 537 | 563 | 472 | 17 | 1,013 |
| 9 | | | 199 | 474 | 15 | 1,048 |
| 10 | | | 11 | 562 | | |

sented in Table II and Fig. 2. These data show convincingly that gestation decreases the milk flow especially after the 4th month of breeding.

The most simple explanation that may be offered concerning the reduction of milk flow by pregnancy is that the demand for nutrients required for growth by the gestating cow reduces by an equivalent amount, the supply of nutrients usually at the disposal of the mammary gland. In other words the nutrients are divided between the needs of the uterus and the mammary gland. This simple explanation is substantiated, first, by the parallelism between the decline

in milk flow and increases in weight of the gestating cow as shown in Fig. 2 (special attention is called to the pause in the body weight and milk secretion curves about the 7th month after breeding); and second, by the fact that the difference in milk flow between the

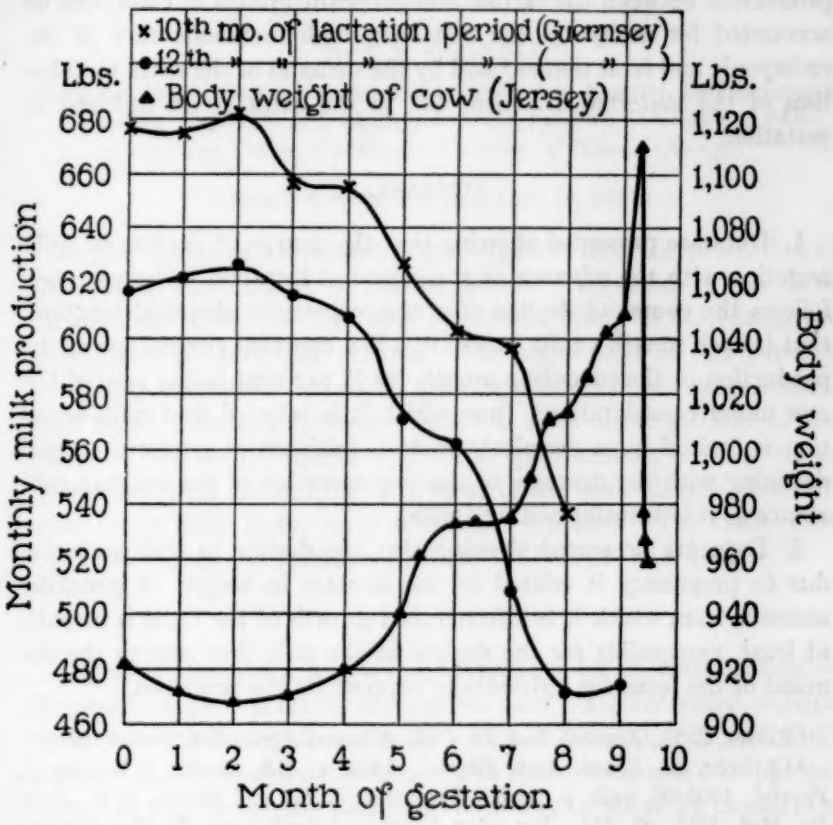


FIG. 2. The effect of the stage of gestation on the milk flow during the 10th and 12th months of the lactation period of Advanced Registry Guernsey cattle, and on the body weight of Jersey cattle. Special attention is called to the pause in the curves during the 6th and 7th months of gestation both in milk secretion and body weight.

pregnant and farrow groups (about 450 lbs. of milk) contains an amount of dry matter which one would expect might be required to produce growth of the calf and to support its life processes while *in*

*utero*. According to Eckles[3] on the dry matter basis, a calf at birth is equivalent to from 110 to 275 lbs. of milk, depending upon the size of the calf and the composition of the milk. The difference between this estimate given by Eckles, and the difference in milk production between the farrow and pregnant groups of cows, can be accounted for easily by the relatively high metabolic rate of the embryonic and fetal tissues,[4] and by the demand of the extra metabolism of the maternal organism due to the extra strain incident to gestation.

## SUMMARY.

1. Data are presented showing that the course of decline of milk secretion with the advance of the period of lactation in farrow cows follows the course of decline of a monomolecular chemical reaction, that is each month's milk production is a constant percentage of the production of the preceding month (94.77 per cent in the case of the cow under consideration), from which it is inferred that milk secretion is limited by a chemical reaction initiated at parturition, and declining with the decrease of the concentration of the limiting substance as it is transformed into milk.

2. Data are presented showing that the decline in milk secretion due to pregnancy is related to the increase in weight of gestating animals, from which it is inferred that growth of the fetus is in part, at least, responsible for the decline in the milk flow due to the demand of the fetus for nutrients to support its life processes.

[3] Eckles, C. H., *Research Bull. 26, Univ. Missouri Agric. Exp. Station*, 1916.

[4] *Cf.* Bohr, C., *Skand. Arch. Physiol.*, 1900, x, 413. Murlin, J. R., *Am. J. Physiol.*, 1908–09, xxiii, p. xxxii. Carpenter, T. M., and Murlin, J. R., *Arch. Int. Med.*, 1911, vii, 184. For other references consult Lusk, G., The elements of the science of nutrition, Philadelphia and London, 3rd edition, 1917, 379–390. Marshall, F. H. A., The physiology of reproduction, London and New York, 1922, Chapter XI.

# STUDIES IN PROTOPLASM POISONING.

## I. Phenols.

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(Received for publication, April 19, 1923.)

In the course of an extended investigation into the protection of wood from the attacks of marine borers,[1,2] opportunity has been afforded the writer to carry on a systematic study of the changes in protoplasm produced by poisons. The major part of this paper is concerned with observations on 8,000 specimens of the small crustacean borer, *Limnoria lignorum*. For the sake of comparison in one phase of this study, results with 7,500 specimens of a fresh water cyprid and with about 1,500 specimens of the brine shrimp, *Artemia*, from Great Salt Lake, have also been included. The data were collected during the summers of 1916, 1917, 1921, and 1922.

### I.

*Limnoria* possesses a number of advantages for an investigation of the present type. It occurs in countless numbers in untreated wooden structures standing in the sea water of the Atlantic, Pacific, and Gulf coasts. It is a small marine isopod 2 to 3 mm. long and 1 to 1½ mm. wide. It may be readily picked up in a drop of water by means of a pipette or medicine dropper.

When it is transferred to sea water from wood in which it is burrowing, and on which it probably subsists, *Limnoria* keeps in constant motion by means of numerous legs and swimmerets. This continuous activity makes it possible to distinguish sharply the moment at which

[1] Shackell, L. F., *Proc. Am. Wood Preservers' Assn.*, 1915, 233; 1916, 124.

[2] Teesdale, C. H., and Shackell, L. F., Engineering News-Record, November 1, 1917.

complete bodily paralysis has been induced by a poison. Again, the first spontaneous movement of the animal after its removal from the toxic solution may be used to delimit a certain stage in its recovery.

Table I embodies a statistical analysis of 4,773 observations on 3,844 specimens of *Limnoria*—covering the actions of phenol, ortho-, meta-, and para-cresol, pyrocatechin, and resorcinol. A discussion of the statistical constants in Table I, to be given later in this paper, will be made clearer by descriptions of three methods by which the experimental data summarized in Table I were obtained.

*First Method.*—The time required by a solution of a phenol to paralyze a given specimen of *Limnoria*, as well as the time taken by the same animal to recover after transfer to fresh sea water, were the objects aimed at in the first method, which was carried out as follows:

Ten freshly collected specimens of *Limnoria* in a Stender dish were freed from sea water by a pipette with capillary tip. They were then covered with 25 cc. of the phenol solution, separated from each other a few millimeters, and kept under continuous observation with the aid of a binocular microscope of low power. The first effect of each phenol investigated was a stimulation, causing spasmodic bendings of the body and contractions of the legs. Locomotion was quickly interfered with, and soon ceased. After a further period of incoordinated contractions in the legs, all movements stopped. As soon as no movement could be detected over the entire body of any specimen, the time was noted within 5 or 10 seconds, and the animal was quickly transferred to a finger-bowl filled with sea water. From the latter it was transferred to a second bowl of sea water and then to a Stender dish half full of water, which contained also a series of glass rings numbered 1 to 10—for identification of each specimen, as well as for convenience and accuracy in obtaining its recovery time. This time was taken at the instant that a specimen showed any spontaneous movement. The animal was then discarded. Six or more repetitions of this work were made with a given phenol solution, each time with fresh lots of ten *Limnoria*.

The experimental method above described was used with the following series in Table I; namely, Nos. 1, 11, 18, 19, 24, 30, 32 to 35, 37, 40, and 48.

*Second Method.*—This method was used to determine whether the degree of correlation between time in a phenol solution and time of recovery was altered by keeping each animal in the poison for twice or three times the time necessary to produce paralysis. The time of paralysis of each specimen was determined for a small group as in the first method. Then, if it was desired—as in Series 2, Table I—to keep each animal in the poison for twice its paralysis time ($2P$), this was done before the animal was transferred to fresh sea water. Otherwise the conduct of the experiment was the same as that in the first method. In this way were obtained the data summarized in the following series in Table I, Nos. 2, 20, 21, and 41.

*Third Method.*—In this method the idea was to determine how the time of recovery was affected when *Limnoria* were kept in a phenol solution for a series of multiples of the mean paralysis time ($\bar{P}$). In this case the paralysis time for each individual was not obtained. The method was carried out as follows: About twenty-five *Limnoria* were put into the phenol solution for a predetermined time—for instance, in Series 3, Table I, they were in 0.125 per cent[3] phenol for 20.50 minutes—that is, for twice the mean time for paralysis in phenol of this concentration.[4] All of the animals were then washed with fresh sea water a number of times and transferred, in lots of five, to saltcellars. The time of initial recovery of each specimen was then noted. This third method, then, differed from the other two in that the recovery time only was obtained for each member of a series of animals which had been in the poison for the same length of time. The results obtained by this method are summarized in Table I, in Series 3 to 10, 12, 17, 22, 23, 25 to 29, 31, 36, 38, 39, 42 to 47, and 49 to 51.

The experimental work was carried on at room temperatures ranging from 27–29°C.

[3] Percentage concentration, as used in this study, is in terms of the number of grams of a phenol dissolved and made up to 100 cc. with water—sea water for *Limnoria*, well water for *Cypris*, and water from Great Salt Lake for *Artemia*.

[4] The mean time for paralysis used here, that is 10.25 minutes, differs from the mean value of 9.95 in Series 1, Table I, because of a difference in the number of determinations from which these two averages were calculated.

| Series No. | Poison. | Concentration. | No. of animals. | Time of immersion. $I$ | | Average time for paralysis. $\bar{P}$ | Average time for recovery. $\bar{R}$ |
|---|---|---|---|---|---|---|---|
| | | *per cent* | | *units* | *min.* | *min.* | *min.* |
| 1 | Phenol. | 0.125 | 70 | | | 9.95 ± 0.15 | 7.42 ± 0.1 |
| 2 | " | 0.125 | 17 | $2\bar{P}$ | | $\bar{I}$ = 21.37 ± 0.79 | 19.53 ± 0.6 |
| 3 | " | 0.125 | 98 | $2\bar{P}$ | 20.50 | | 18.98 ± 0.2 |
| 4 | " | 0.125 | 93 | $4\bar{P}$ | 41.00 | | 33.26 ± 0.3 |
| 5 | " | 0.125 | 97 | $8\bar{P}$ | 82.00 | | 46.09 ± 0.2 |
| 6 | " | 0.125 | 92 | $12\bar{P}$ | 123.00 | | 58.43 ± 0.6 |
| 7 | " | 0.125 | 96 | $16\bar{P}$ | 164.00 | | 74.35 ± 0.9 |
| 8* | " | 0.125 | 99 | $6\bar{P}$ | 61.50 | | 36.39 ± 0.2 |
| 9* | " | 0.125 | 99 | $10\bar{P}$ | 102.00 | | 45.03 ± 0.3 |
| 10* | " | 0.125 | 99 | $14\bar{P}$ | 143.00 | | 57.21 ± 0.5 |
| 11 | " | 0.25 | 70 | | | 8.34 ± 0.20 | 14.99 ± 0.3 |
| 12 | " | 0.25 | 100 | $2\bar{P}$ | 17.00 | | 36.12 ± 0.1 |
| 13 | " | 0.25 | 99 | $4\bar{P}$ | 34.00 | | 59.36 ± 0.4 |
| 14 | " | 0.25 | 96 | $6\bar{P}$ | 51.00 | | 85.48 ± 0.6 |
| 15 | " | 0.25 | 19 | $8\bar{P}$ | 68.00 | | 110.89 ± 0.6 |
| 16* | " | 0.25 | 99 | $3\bar{P}$ | 25.67 | | 42.30 ± 0.3 |
| 17* | " | 0.25 | 92 | $5\bar{P}$ | 42.83 | | 65.43 ± 0.6 |
| 18 | " | 0.375 | 70 | | | 7.54 ± 0.13 | 26.55 ± 0.3 |
| 19 | " | 0.50 | 70 | | | 6.82 ± 0.13 | 31.79 ± 0.4 |
| 20 | " | 0.50 | 30 | $2P$ | | $\bar{I}$ = 16.78 ± 0.44 | 84.13 ± 1.5 |
| 21 | " | 0.50 | 27 | $3P$ | | $\bar{I}$ = 23.21 ± 0.53 | 113.73 ± 3.1 |
| 22 | " | 0.50 | 91 | $2\bar{P}$ | 15.33 | | 69.52 ± 0.3 |
| 23 | " | 0.50 | 69 | $3\bar{P}$ | 23.00 | | 129.24 ± 1.1 |
| 24 | Ortho-cresol. | 0.125 | 69 | | | 9.12 ± 0.19 | 17.49 ± 0.4 |
| 25* | " | 0.125 | 100 | $2\bar{P}$ | 18.42 | | 46.82 ± 0.2 |
| 26* | " | 0.125 | 98 | $3\bar{P}$ | 27.58 | | 66.36 ± 0.3 |
| 27* | " | 0.125 | 100 | $4\bar{P}$ | 36.83 | | 82.34 ± 0.4 |
| 28* | " | 0.125 | 99 | $5\bar{P}$ | 46.00 | | 102.48 ± 0.4 |
| 29* | " | 0.125 | 95 | $6\bar{P}$ | 55.00 | | 119.49 ± 0.6 |
| 30 | " | 0.25 | 66 | | | 8.26 ± 0.16 | 51.30 ± 0.3 |
| 31 | " | 0.25 | 99 | $2\bar{P}$ | 15.00 | | 89.02 ± 0.4 |
| 32 | Meta-cresol. | 0.125 | 69 | | | 7.53 ± 0.14 | 20.19 ± 0.4 |
| 33 | " | 0.25 | 68 | | | 7.15 ± 0.16 | 47.17 ± 0.3 |
| 34 | Para-cresol. | 0.125 | 68 | | | 9.83 ± 0.23 | 20.60 ± 0.3 |
| 35 | " | 0.25 | 67 | | | 7.32 ± 0.19 | 43.58 ± 0.3 |
| 36 | " | 0.25 | 100 | $2\bar{P}$ | 15.00 | | 88.42 ± 0.3 |
| 37 | Pyrocatechin. | 0.25 | 83 | | | 30.35 ± 0.53 | 11.15 ± 0.2 |
| 38 | " | 0.25 | 72 | $2\bar{P}$ | 56.00 | | 32.24 ± 0.5 |
| 39 | " | 0.25 | 93 | $4\bar{P}$ | 112.00 | | 65.83 ± 1.0 |
| 40 | " | 0.50 | 79 | | | 16.36 ± 0.34 | 16.22 ± 0.4 |
| 41 | " | 0.50 | 16 | $3P$ | | $\bar{I}$ = 37.41 ± 1.33 | 67.23 ± 2.0 |
| 42 | " | 0.50 | 97 | $2\bar{P}$ | 32.00 | | 44.89 ± 0.3 |
| 43 | " | 0.50 | 67 | $3\bar{P}$ | 48.00 | | 83.75 ± 1.0 |
| 44 | " | 0.50 | 30 | $4\bar{P}$ | 65.00 | | 128.15 ± 1.9 |
| 45* | Resorcinol. | 0.25 | 50 | $2\bar{P}$ | 70.00 | | 19.82 ± 0.4 |
| 46* | " | 0.25 | 49 | $4\bar{P}$ | 140.00 | | 29.92 ± 0.2 |
| 47* | " | 0.25 | 46 | $6\bar{P}$ | 210.00 | | 40.61 ± 0.6 |
| 48 | " | 0.50 | 80 | | | 19.33 ± 0.34 | 16.13 ± 0.4 |
| 49 | " | 0.50 | 73 | $2\bar{P}$ | 38.00 | | 38.61 ± 0.2 |
| 50 | " | 0.50 | 59 | $3\bar{P}$ | 57.00 | | 52.04 ± 0.3 |
| 51 | " | 0.50 | 60 | $4\bar{P}$ | 76.00 | | 66.46 ± 1.0 |

* These series were carried out in 1917; all other series were carried out in 1916.

| Standard deviation of paralysis. $S.D._P$ | Standard deviation of recovery. $S.D._R$ | Coefficient of variation—paralysis. $C.V._P$ | Coefficient of variation—recovery. $C.V._R$ | Correlation between paralysis and recovery. $r_{PR}$ |
|---|---|---|---|---|
| *min.* | *min.* | | | |
| 1.83 ± 0.10 | 1.94 ± 0.11 | 18.39 ± 1.08 | 26.15 ± 1.59 | 0.530 ± 0.058 |
| $D_I$ = 4.84 ± 0.56 | 4.19 ± 0.49 | $C.V._I$ = 22.65 ± 2.75 | 21.45 ± 2.59 | $r_{IR}$ = 0.880 ± 0.037 |
| | 2.45 ± 0.12 | | 12.91 ± 0.63 | |
| | 2.21 ± 0.11 | | 6.64 ± 0.33 | |
| | 3.96 ± 0.19 | | 8.59 ± 0.42 | |
| | 8.47 ± 0.42 | | 14.50 ± 0.74 | |
| | 14.27 ± 0.69 | | 19.19 ± 0.97 | |
| | 3.28 ± 0.16 | | 9.01 ± 0.44 | |
| | 4.95 ± 0.24 | | 10.99 ± 0.53 | |
| | 8.71 ± 0.42 | | 15.22 ± 0.74 | |
| 2.48 ± 0.14 | 4.21 ± 0.24 | 29.74 ± 1.83 | 28.09 ± 1.71 | 0.744 ± 0.036 |
| | 2.84 ± 0.14 | | 7.86 ± 0.38 | |
| | 5.91 ± 0.28 | | 9.96 ± 0.48 | |
| | 8.89 ± 0.43 | | 10.40 ± 0.51 | |
| | 2.69 ± 0.29 | | 2.43 ± 0.27 | |
| | 4.44 ± 0.21 | | 10.50 ± 0.51 | |
| | 6.08 ± 0.30 | | 9.29 ± 0.47 | |
| 1.63 ± 0.09 | 4.46 ± 0.25 | 21.62 ± 1.28 | 16.80 ± 0.99 | 0.821 ± 0.026 |
| 1.60 ± 0.09 | 5.93 ± 0.34 | 23.46 ± 1.40 | 18.65 ± 1.09 | 0.927 ± 0.011 |
| $D_I$ = 3.57 ± 0.31 | 15.58 ± 1.36 | $C.V._I$ = 21.28 ± 1.93 | 18.52 ± 1.66 | $r_{IR}$ = 0.938 ± 0.015 |
| $D_I$ = 4.12 ± 0.38 | 29.06 ± 2.67 | $C.V._I$ = 17.75 ± 1.68 | 25.55 ± 2.49 | $r_{IR}$ = 0.836 ± 0.039 |
| | 3.42 ± 0.17 | | 4.92 ± 0.25 | |
| | 22.76 ± 1.31 | | 17.61 ± 1.04 | |
| 2.34 ± 0.13 | 4.89 ± 0.28 | 25.66 ± 1.56 | 27.96 ± 1.73 | 0.696 ± 0.042 |
| | 3.78 ± 0.18 | | 8.07 ± 0.39 | |
| | 4.19 ± 0.20 | | 6.31 ± 0.30 | |
| | 9.18 ± 0.44 | | 11.15 ± 0.54 | |
| | 5.98 ± 0.29 | | 5.84 ± 0.28 | |
| | 7.14 ± 0.35 | | 5.98 ± 0.29 | |
| 1.96 ± 0.12 | 10.14 ± 0.60 | 23.73 ± 1.46 | 19.77 ± 1.21 | 0.779 ± 0.033 |
| | 6.53 ± 0.31 | | 7.34 ± 0.35 | |
| 1.69 ± 0.10 | 5.16 ± 0.30 | 22.44 ± 1.35 | 25.56 ± 1.56 | 0.735 ± 0.037 |
| 1.98 ± 0.11 | 10.95 ± 0.63 | 27.69 ± 1.71 | 23.21 ± 1.41 | 0.895 ± 0.016 |
| 2.82 ± 0.16 | 6.03 ± 0.35 | 28.69 ± 1.79 | 29.27 ± 1.83 | 0.794 ± 0.030 |
| 2.27 ± 0.13 | 9.19 ± 0.54 | 31.01 ± 1.97 | 21.09 ± 1.28 | 0.748 ± 0.036 |
| | 5.63 ± 0.27 | | 6.37 ± 0.30 | |
| 7.17 ± 0.38 | 3.78 ± 0.20 | 23.62 ± 1.30 | 33.90 ± 1.97 | 0.490 ± 0.056 |
| | 6.66 ± 0.37 | | 20.66 ± 1.21 | |
| | 14.57 ± 0.72 | | 22.13 ± 1.15 | |
| 4.52 ± 0.24 | 5.59 ± 0.30 | 27.63 ± 1.58 | 34.46 ± 2.05 | 0.579 ± 0.050 |
| $D_I$ = 7.86 ± 0.94 | 15.83 ± 1.89 | $C.V._I$ = 21.01 ± 2.60 | 23.55 ± 2.95 | $r_{IR}$ = 0.917 ± 0.027 |
| | 10.00 ± 0.48 | | 22.28 ± 1.13 | |
| | 12.11 ± 0.71 | | 14.46 ± 0.86 | |
| | 16.09 ± 1.40 | | 12.56 ± 1.11 | |
| | 4.60 ± 0.31 | | 23.21 ± 1.64 | |
| | 3.75 ± 0.26 | | 12.53 ± 0.87 | |
| | 4.80 ± 0.34 | | 11.82 ± 0.84 | |
| 4.45 ± 0.24 | 5.48 ± 0.29 | 23.02 ± 1.29 | 33.97 ± 2.01 | 0.591 ± 0.049 |
| | 8.95 ± 0.50 | | 23.18 ± 1.36 | |
| | 8.65 ± 0.54 | | 16.61 ± 1.06 | |
| | 11.70 ± 0.72 | | 17.60 ± 1.12 | |

II.

Table I may now be considered in some detail. In Column 1, the experimental series which are starred were carried out in 1917; all other series in 1916. In certain of these series (Nos. 8, 9, 10, 16, and 17) the experiments were carried out with the idea that the data obtained might be incorporated into the graphs based on the 1916 data. But it was found that a seasonal variation had occurred, and that the *Limnoria* were definitely less susceptible to poisoning in 1917 than in 1916. In Column 4 is given the number of animals observed in a given series. In Column 5 is given the number of paralysis-time units during which the animals of a given series were immersed in the poison. The equivalent in minutes has been put in Column 6. An exception to this last is to be noted in the case of experiments carried out by the second method. Here the time of immersion in a given poison is indicated by some multiple of $P$—the paralysis time for each individual specimen. The average of this time of immersion, or multiple of $P$, was called $\bar{I}$, and has been put in Column 7. If $\bar{I}$ be divided by the coefficient of $P$, the result obtained will be the average time for paralysis for that series. Standard deviations, coefficients of variation, and coefficients of correlation are given in Columns 9 to 13.

Attention may be directed to certain significant details of Table I. These are: (*a*) the relationships between paralysis and recovery times for the several phenols which were investigated; (*b*) the changes in variability of the recovery time values, with increase in the time of immersion in a given preparation; and (*c*) the rise in correlation between time for paralysis and time for recovery with increase in concentration or increase in the time of immersion. These points are considered in the remaining paragraphs of this section.

In the case of phenol (Table I, Series 1, 11, 18, and 19, Columns 7 and 9), it will be seen that an increase in concentration from 0.125 per cent to 0.5 per cent reduces the mean time for paralysis from 9.95 minutes to 6.82 minutes. The mean time of recovery increases, however, practically in the same ratio as the increase in concentration; that is, from 7.42 minutes for 0.125 per cent to 31.79 minutes for 0.5 per cent. In the case of each of the cresols (Series 24, 30, 32 to 35)

doubling the concentration reduces the mean time of paralysis relatively slightly, whereas it more than doubles the time required for beginning recovery. With pyrocatechin (Series 37 and 40) the case is still different. Here the mean time for paralysis is nearly halved by doubling the concentration, while the mean time for beginning recovery is increased by less than 50 per cent.

The above facts seem to permit of two conclusions. The first is that paralysis in *Limnoria* is not determined at the moment that a particular concentration of a phenol is reached in the animal's tissues, but that paralysis is a certain stage in a process (or series of processes) set into motion at the instant the poison first enters the animal. The second conclusion is that the relative changes in paralysis and recovery times with changes in concentration of a phenol are closely related to the relative solubilities of the phenol in water and lipins, respectively. Further evidence in support of these conclusions will be considered later.

A study of the variabilities of recovery time (Column 12) discloses that there is a marked reduction in the coefficient of variation as the time of immersion is increased. When the time in the poison is increased beyond a certain point, however, the variability seems to show a secondary rise. It is to be noted that in all series in which the coefficient of variation is low, the third experimental method, previously described, was used; that is, all animals were kept in the poison for the same length of time. The recovery time was then obtained for each animal. The matter of keeping animals in such experiments in the poison for the same length of time is probably the principal factor in lowering the variability; for animals which were paralyzed quickly would have their recovery times lengthened relative to those animals which were only slowly paralyzed. In this way the range of recovery time values would be narrowed.

The coefficients of correlation between time of paralysis and recovery (Column 13) are of considerable significance. These coefficients for 0.125, 0.25, 0.375, and 0.5 per cent phenol are, respectively, 0.530, 0.744, 0.821, and 0.927. Ortho-cresol, meta-cresol, and pyrocatechin show similar rises in this coefficient with rise in concentration. Para-cresol is the only exception.

This increase in correlation with increase in concentration of a phenol indicates that perfect correlation (= unity) between time of paralysis and time of recovery should be approximated with a concentration of the phenol with which the animals of a given series were virtually in equilibrium by the time they became paralyzed.

That the correlation between immersion time and recovery time tends to approximate unity when a number of animals are kept in a given phenol solution for twice or three times their respective paralysis times is shown for 0.125 per cent phenol in Series 1 and 2; and for 0.5 per cent pyrocatechin in Series 40 and 41. For example, when *Limnoria* are removed from 0.125 per cent phenol to a normal environment at the moment they are paralyzed, this coefficient is found to be 0.530. When, now, *Limnoria* are allowed to remain in the phenol solution for an additional time equal to the time which was required for paralysis, this coefficient rises to 0.880. The rise in this coefficient for 0.5 per cent pyrocatechin is from 0.579 to 0.917 (Series 40 and 41). From this it may be inferred that when *Limnoria* are kept in a phenol solution after equilibrium with the solution has been attained, the time of recovery will bear a simple linear relationship to the time of immersion—at least as long as initial recovery may be fairly sharply defined. This inference has been verified experimentally.

III.

If, for a given phenol solution, the values of $\bar{R}$, the average time for beginning recovery, be plotted as ordinates against corresponding values of $t$, the time the animals have been in the poison, it is found that practically all of the plotted points lie on straight lines (Figs. 1 to 7). In each of these figures the straight line is the graph of the equation to be found just above the axis of abscissæ. The values for the points located by circles were not used in calculating the constants of the respective equations. An explanation, consonant with the fact that these aberrant values are seen only at the beginning of poisoning, is that the animals had not yet reached equilibrium with the poison. This presumption is strengthened by the fact that with 0.125 per cent phenol (Fig. 1) two values required to be left out in the calculation of the constants; with 0.25 per cent phenol (Fig. 2) but one value was left out; while with 0.5 per cent phenol (Fig. 3)

all values were included in the calculation. In other words, the higher the concentration gradient, the more nearly would animals be in equilibrium with the poison at the time of paralysis.

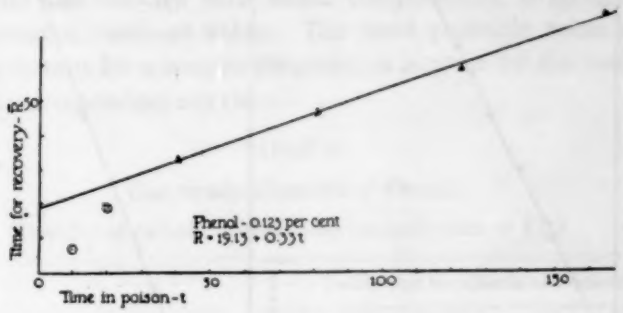



FIG. 1.

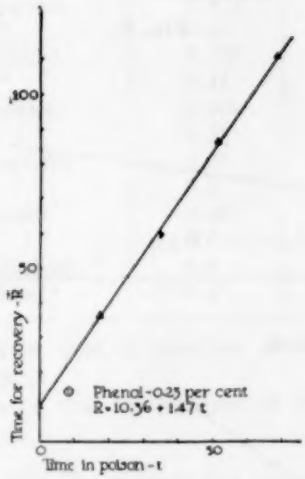



FIG. 2.

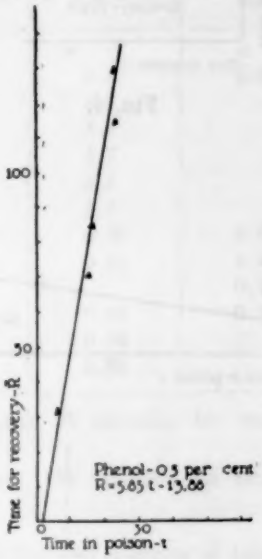



FIG. 3.

It may be well to point out that each of the ordinate values in Figs. 1 to 7 is the mean of a considerable number of observations. The four points, for instance, which lie along the straight line in Fig. 1, are based upon 378 measurements of recovery time, made upon as many different animals.

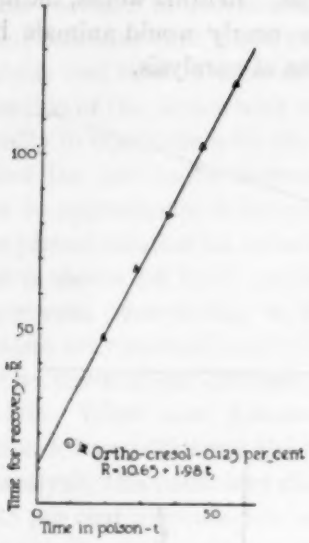



FIG. 4.

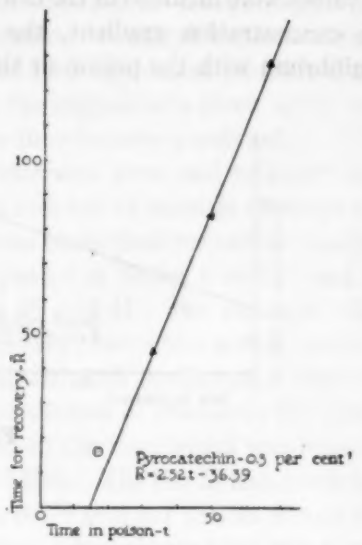



FIG. 5.

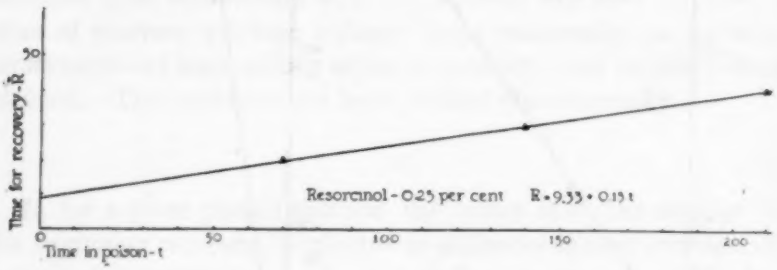



FIG. 6.

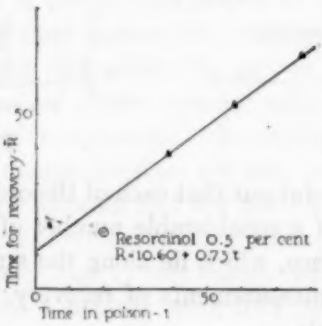



FIG. 7.

Figs. 1 to 7 show clearly that, within rather wide limits, the progress of poisoning of *Limnoria* by each of four different phenols is a simple linear function of the time of immersion in the poison. From this it follows that the velocity with which the poisoning is going on oscillates about a constant value. The most probable value of this velocity constant for a specific preparation is given by the coefficient of $t$ in the corresponding equation.

TABLE II.

*Comparative Toxicities of Phenols.*

(Toxicity of carbolic acid of same concentration = 1.0.)

| Phenol. | Concentration. | Toxicity when the following are compared: | | |
|---|---|---|---|---|
| | | Respective paralysis—time values. | Respective ratios of $\overline{R} : \overline{P}$ | Coefficients of $t$ in respective equations. |
| | *per cent* | | | |
| Ortho-cresol. | 0.125 | 1.1 | 2.6 | 6.0 |
| " | 0.25 | 1.0 | 3.5 | |
| Meta-cresol. | 0.125 | 1.3 | 3.6 | |
| " | 0.25 | 1.2 | 3.7 | |
| Para-cresol. | 0.125 | 1.0 | 2.8 | |
| " | 0.25 | 1.1 | 3.3 | |
| Pyrocatechin. | 0.25 | 0.33 | 0.20 | 0.41 |
| " | 0.5 | 0.42 | 0.21 | 0.43 |
| Resorcinol. | 0.25 | | | 0.10 |
| " | 0.5 | 0.35 | 0.18 | 0.10 |
| Hydroquinone.* | 0.5 | 0.08 | 0.04 | |
| Pyrogallol.† | 0.5 | 0.05 | 0.04 | |

* Average time for paralysis (10 observations), 88 minutes; for recovery, 16.3 minutes.

† Average time for paralysis (9 observations), 151 minutes; for recovery, 26 minutes.

In Table II are given the toxicity coefficients of a series of phenols, in units of phenol (carbolic acid) toxicity for the same concentration, obtained by comparing (*a*) paralysis time values; (*b*) ratios of recovery to paralysis times; and (*c*) linear velocity constants (coefficients of $t$). The ratios of the respective velocity constants probably yield the most reliable values for the relative toxicities of different phenols, or of different concentrations of the same phenol. From

the toxicity coefficients in Table II, the phenols used in this study may be listed in the descending order of toxicity as follows: The cresols, carbolic acid, pyrocatechin, resorcinol, hydroquinone, pyrogallol.

It seems worthy of remark that when the concentration is doubled, the velocity of poisoning is increased four times or more. For instance, 0.25 per cent phenol is $\frac{1.47}{0.33}$, or 4.5, times as poisonous as 0.125 per cent phenol. One-half per cent phenol is $\frac{5.85}{1.47}$, or 4.0, times as poisonous as 0.25 per cent. One-half per cent pyrocatechin is 4.2 times as poisonous as 0.25 per cent, while 0.5 per cent resorcinol is 4.9 times as toxic as 0.25 per cent. It may, therefore, be said that the velocity of poisoning is nearly proportional to the square of the concentration.

IV.

Since it might be objected that recovery from paralysis would not offer an adequate criterion of the progress of poisoning, further experimental work was carried out in the summers of 1921 and 1922. The criteria of toxicity adopted consisted of the percentages of animals which died after stated periods in a phenol solution. The preparations used were 0.25 and 0.5 per cent phenol. The animals used in this phase of the study were *Limnoria*, *Cypris*, and *Artemia*. Substantially the same experimental procedure was used with *Cypris* and *Artemia* as is described below for *Limnoria*.

In making the following experiments, 100 or more active specimens of *Limnoria* were put into the phenol solution for a definite length of time. At the end of this period the poison was decanted, the animals were washed repeatedly with fresh sea water, and finally transferred to a finger-bowl full of sea water. After 18 hours the number of animals which had died was determined. Numerous control experiments showed that the mortality rate of active *Limnoria* within the first 24 hours after removal from their burrows was less than 1 per cent.

A composite of twenty experiments with *Limnoria*, carried out as just described, is given in Table III. In Fig. 8 is the graph obtained

by plotting the values in Column 3 of Table III as ordinates against the corresponding time periods in Column 1. With the exception of the two extreme values—at 12 minutes and 33 minutes—the plotted points lie practically on a straight line. The most probable numerical value for the slope of this line, using the six values from 15 to 30 minutes, is 5.62—in quite good agreement with the velocity coefficient of 5.85 for 0.5 per cent phenol (Fig. 3), obtained by the use of an

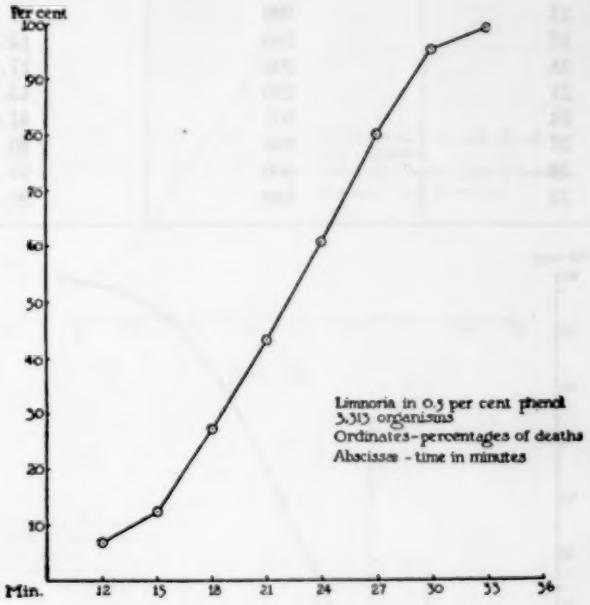



FIG. 8.

entirely different criterion of toxicity. Since this finding appears to be of fundamental significance, a more detailed analysis of the graph in Fig. 8 seems necessary.

The S-shaped curve in Fig. 8 is not an anomalism. Curves of this type have been obtained by Brooks[5] for the course of hemolysis of erythrocytes after radiation from a mercury vapor arc in quartz; and by Bovie and Hughes[6] for the rate of cytolysis of *Paramecia* after

[5] Brooks, S. C., *J. Gen. Physiol.*, 1918–19, i, 61.

[6] Bovie, W. T., and Hughes, D. M., *J. Gen. Physiol.*, 1918–19, i, 323.

fluorite radiation. The experiments of the writer with *Cypris* in 0.5 per cent phenol (Fig. 9) and with *Artemia* in 0.25 per cent phenol

TABLE III.

*Limnoria in 0.5 Per Cent Phenol.*

| Time in poison. | No. of *Limnoria* used. | Animals killed. |
|---|---|---|
| *min.* | | *per cent* |
| 12 | 100 | 7.0 |
| 15 | 200 | 12.5 |
| 18 | 306 | 27.2 |
| 21 | 200 | 43.5 |
| 24 | 907 | 61.0 |
| 27 | 800 | 80.1 |
| 30 | 600 | 95.3 |
| 33 | 200 | 99.0 |

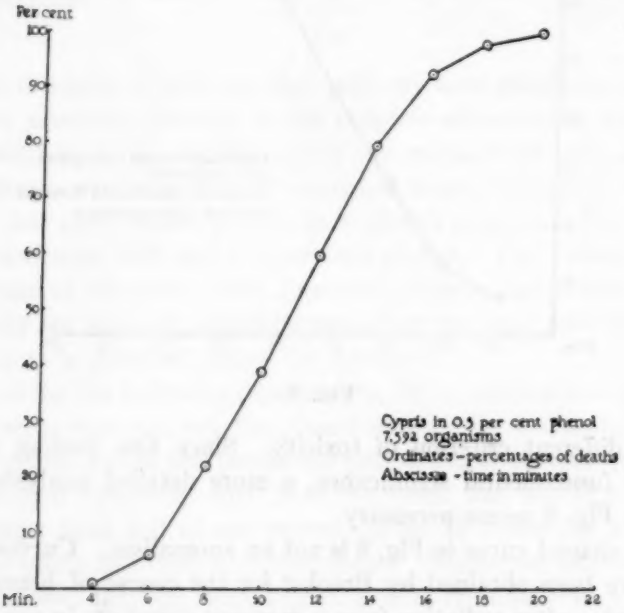



FIG. 9.

(Fig. 10) have yielded curves of the same type. In fact, from the results of a few experiments with mineral acids, it is not improbable

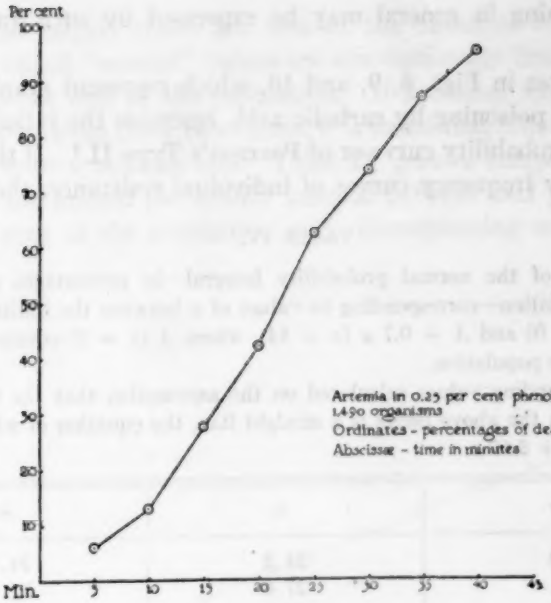
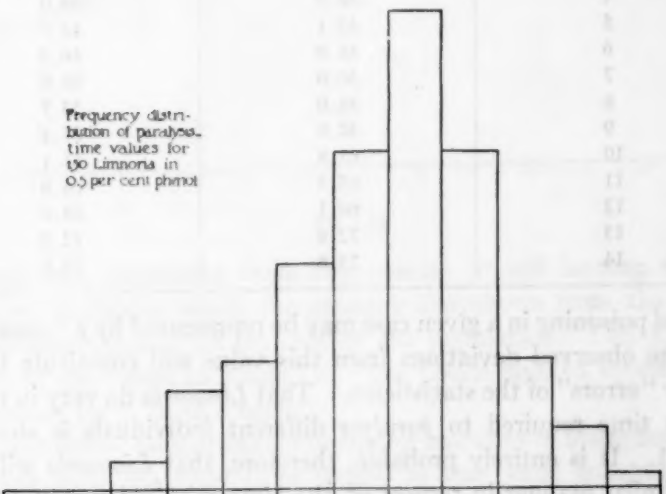



FIG. 10.




| Class values in minutes | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|
| Frequencies | 2 | 7 | 9 | 16 | 24 | 34 | 24 | 9 | 4 | 1 |

FIG. 11.

that poisoning in general may be expressed by such an S-shaped curve.

The curves in Figs. 8, 9, and 10, which represent graphically the progress of poisoning by carbolic acid, resemble the integral form of a normal probability curve or of Pearson's Type II.[7] If these graphs are actually frequency curves of individual resistance, then the progress of poisoning in a given case may be represented by a "constant," and the observed deviations from this value will constitute the ordinary "errors" of the statistician. That *Limnoria* do vary in respect of the time required to *paralyze* different individuals is shown in Fig. 11. It is entirely probable, therefore, that *Limnoria* will vary in a similar manner in respect of the time required to *kill* different individuals.

TABLE IV.

$y_1$ = values of the normal probability integral—in percentages of the total population—corresponding to values of $x$ between the limits $A - 0.7\,\sigma$ ($x = 0$) and $A + 0.7\,\sigma$ ($x = 14$), where $A$ ($x = 7$) equals 50 per cent of the population.

$y_2$ = corresponding values calculated on the assumption that the function between the above limits is a straight line, the equation of which is $y_2 = 24.2 + 3.69\,x$.

| $x$ | $y_1$ | $y_2$ |
|---|---|---|
| 0 | 24.2 | 24.2 |
| 1 | 27.4 | 27.9 |
| 2 | 30.9 | 31.6 |
| 3 | 34.5 | 35.3 |
| 4 | 38.2 | 39.0 |
| 5 | 42.1 | 42.7 |
| 6 | 46.0 | 46.3 |
| 7 | 50.0 | 50.0 |
| 8 | 54.0 | 53.7 |
| 9 | 57.9 | 57.4 |
| 10 | 61.8 | 61.1 |
| 11 | 65.5 | 64.8 |
| 12 | 69.1 | 68.5 |
| 13 | 72.6 | 72.2 |
| 14 | 75.8 | 75.8 |

[7] Davenport, C. B., Statistical methods with special reference to biological variation, New York, 3rd edition, 1914, 30.

Since the largest errors are seen at the extremes of a variation curve, so called "normal" values are conventionally limited between 25 and 75 per cent of the population. If a normal variation curve be plotted between these two values, it is found that it does not depart seriously from a straight line. Table IV gives a comparison of the values of the normal probability integral between 24.2 per cent and 75.8 per cent of the population with corresponding values for the

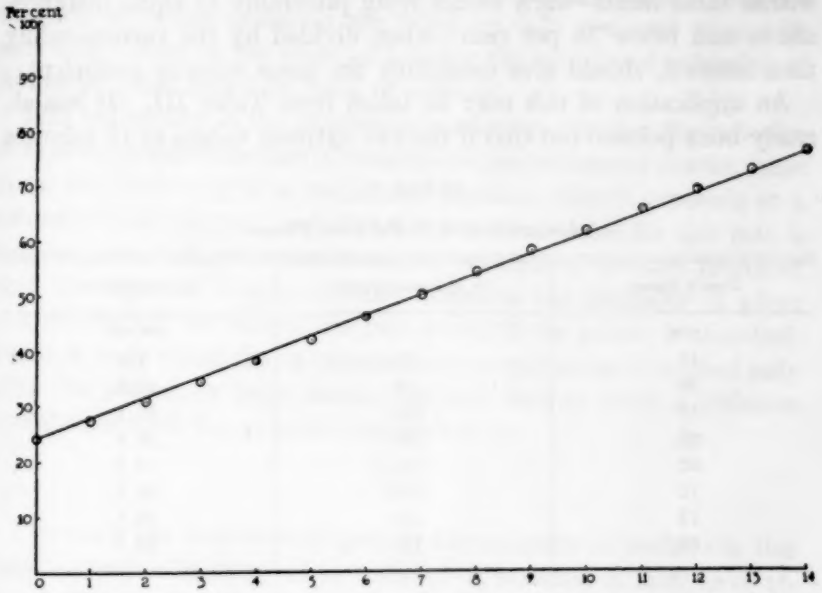



FIG. 12.

straight line connecting these two points. It will be seen that the greatest deviation which the straight line shows from the normal probability values is 0.8 per cent of the population. Fig. 12 is the graph of the values in Table IV. The points marked by circles are the values for $y_1$ in this table.

Quantitative measurements involving reactions of living matter are affected with relatively large errors, so that valid conclusions can be drawn only from large numbers of observations. It seems, then, that the velocity of poisoning by any agent may be obtained most accurately and with a minimum of experimentation by the following method.

A time, $t_1$, is found at which about 25 per cent of the organisms are killed. Another time, $t_2$, is also found at which about 75 per cent of the organisms are killed. The numerical value for the velocity of killing may then be obtained by dividing the difference between the higher and lower percentages by $t_2 - t_1$. Furthermore, since values intermediate between 25 and 75 per cent have been shown to lie virtually on a straight line, the difference between any pairs of values within these limits—such values lying preferably at equal distances above and below 50 per cent—when divided by the corresponding time interval, should give essentially the same velocity coefficient.

An application of this may be taken from Table III. It has already been pointed out that if the two extreme values at 12 minutes

TABLE V.

*Limnoria in 0.25 Per Cent Phenol.*

| Time in poison. | No. of *Limnoria* used. | Animals killed. |
|---|---|---|
| *min.* | | *per cent* |
| 45 | 112 | 1.8 |
| 50 | 110 | 10.0 |
| 55 | 110 | 19.1 |
| 60 | 111 | 36.9 |
| 65 | 111 | 43.2 |
| 70 | 109 | 46.8 |
| 75 | 110 | 59.1 |
| 80 | 111 | 68.5 |

and 33 minutes be eliminated, the most probable value for the rate of poisoning of *Limnoria* in 0.5 per cent phenol is 5.62. If, however, (using the figures in Table III) the nearest value to 25 per cent, namely 27.2, be subtracted from the nearest value to 75 per cent, namely 80.1, and the result divided by 9—the difference between the corresponding $t$ values—a quotient of 5.88 is obtained. Again, if the difference between 61.0 per cent and 43.5 per cent be divided by the time which has elapsed, namely 3 minutes, a velocity coefficient of 5.83 is obtained. These values of 5.88 and 5.83 for the rate at which *Limnoria* are *killed* by 0.5 per cent phenol, are in quite remarkable agreement with the value of 5.85 for the velocity of poisoning of *Limnoria* with the same concentration of phenol when based on time of *recovery*.

Similar results have been obtained with 0.25 per cent phenol, the data for which are given in Table V. If the same method of calculating the velocity coefficient be used as has been done for 0.5 per cent phenol, two values can be obtained. The first is obtained by dividing the difference between 36.9 and 68.5 by 20—the difference between the corresponding *t* periods—yielding a numerical value of 1.58. If the intermediate values for 65 and 75 minutes, respectively, are used, a velocity coefficient of 1.59 is obtained. These values for the rate at which *Limnoria* are *killed* by 0.25 per cent phenol lie reasonably close to the value of 1.47 for the velocity of poisoning of *Limnoria* when based on time of *recovery*.

This uniformity in the progress of poisoning, whether the criterion of toxicity is based on time of recovery or percentages of deaths, must mean that poisoning is a continuous function, that it proceeds at a virtually constant rate, and that the numerical value for this rate is independent of the criterion of toxicity which may be used, provided that the criterion chosen actually measures the intensity of effect of a poison and not merely the rate at which the poison is absorbed. For it is to be noted that a constant rate of poisoning is reached only after the poison, in large excess, has had time to reach a diffusion equilibrium with the animals immersed in it.

## V.

Poisoning has been looked upon by the majority of workers in this field as essentially chemical in nature. A number, in fact, have attempted to apply the monomolecular formula to the killing of bacteria and other organisms. The reader who is interested in this phase of the subject will find good bibliographies in the papers by Bowers,[8] Brooks,[9] and Fairhall.[10] The protest voiced by Brooks against the stereotyped application of the monomolecular formula is particularly timely. A similar stand has been taken by Rideal and Rideal.[11] The writer believes that the time is ripe for establishing a reorientation with respect to chemical and physical explanations

[8] Bowers, E. B., *Illinois Biological Monographs*, 1917, iv, 127.
[9] Brooks, S. C., *J. Gen. Physiol.*, 1918–19, i, 61.
[10] Fairhall, L. T., *Mil. Surg.*, 1922, l, 295.
[11] Rideal, S., and Rideal, E. K., Disinfection, London, 1922, 299.

of phenomena peculiar to living matter. It will scarcely be debated that anything like clear distinctions between chemical and physical mechanisms will come only through arbitrary definitions laid down by those who are in a position to give such definitions reasonable permanence among our scientific concepts. Until such a time comes, nothing can be gained by attempting to put certain vital phenomena exclusively into one category or the other.

The investigations of Brooks,[9] Küster and Rothaub,[12] Cooper,[13] Traube,[14] Clowes and Keith,[15] and Traube and Somogyi,[16] are sufficient to show that a strictly chemical (monomolecular) interpretation of poisoning (or disinfection) offers an entirely inadequate description of the mechanisms involved. The results of the writer show that a straight line describes the progress of poisoning by phenols with precision when a vital factor, namely the variability in resistance of individual organisms, is taken into account. Now, the concept of a monomolecular reaction involves a change in rate from instant to instant—a concept not in harmony with any linear function; that is, one proceeding at a constant rate. The writer is therefore of the opinion that only by taking into consideration such factors as cell permeability, osmosis, surface tension and adsorption, viscosity, electrical double layers, distribution coefficients between lipins and water, swelling, coalescence, and dispersion, in addition to factors which are unquestionably chemical in nature, can poisoning be adequately described.

Nevertheless, in considering the possible mechanisms of poisoning by phenols, there are two of these factors on which attention may be focussed for the time being. These are: (1) the distribution of a phenol between the water and lipins of protoplasm; and (2) the aggregation or coalescence of protein particles. In this connection it is worth noting that the monohydroxyphenols—carbolic acid and the cresols—are more soluble in many substances of lipoid character than they are in water, and that an increase in the number of hydroxyl

[12] Küster, E., and Rothaub, *Z. Hyg.*, 1913, lxxiii, 205.
[13] Cooper, E. A., *Biochem. J.*, 1912–13, vii, 175.
[14] Traube, J., *Biochem. Z.*, 1919, xcviii, 177.
[15] Clowes, G. H. A., and Keith, L. G., *J. Biol. Chem.*, 1920, xli, p. xxxvii.
[16] Traube, J., and Somogyi, R., *Biochem. Z.*, 1921, cxx, 90.

groups in the phenols is accompanied by increased solubility in water. Furthermore, carbolic acid and the cresols are more powerful flocculants of proteins than the di- or trioxyphenols. The latter, however, are more easily oxidized and disintoxicated than the monoxyphenols. A further fact bearing on the above points, which was regularly seen in the writer's experiments, was that in dead animals the tissues were quite opaque—in marked contrast to the translucency seen in the tissues of living animals. This seems to indicate that death of tissue cells is determined by a certain grade of aggregation of the cell proteins.

A tentative theory of poisoning by phenols may now be stated, based principally upon the writer's experimental findings, that the markedly lipin-soluble monoxyphenols (carbolic acid, cresols) cause relatively rapid paralysis, from which recovery is relatively slow; whereas the markedly water-soluble di- and trioxyphenols (pyrocatechin, resorcinol, hydroquinone, pyrogallol) take a considerable time to produce paralysis, from which the recovery is rapid.

As a working concept of the general lipin : protein relationship in unpoisoned protoplasm, let it be assumed that a factor in the maintenance of the normal dispersity of tissue proteins is the adsorption of ultramicroscopic lipins at protein/water interfaces. Now let a phenol, which is relatively slightly soluble in water, but quite soluble in lipins—such as carbolic acid or one of the cresols—diffuse from its aqueous solution into the tissue. The accumulation of the phenol in the lipoid adsorption layer, which may reach a concentration several times that in the water, must at least disturb the preexisting lipin: protein relationship. This disturbance will conceivably facilitate the aggregation (flocculation) of the tissue proteins.

Assume, now, a second case in which the phenol is very soluble in water; that is, one of the di- or trioxyphenols. Here the accumulation of the phenol in the lipin phase can scarcely exceed, if it even reaches, that in the aqueous phase. The result must be a less rapid aggregation of the tissue proteins with consequent slower poisoning and death.

The rate at which a phenol is disintoxicated by protoplasm must eventually receive its share of attention in any comprehensive description of poisoning by this class of compounds. For the present

it may be pointed out that the chemical instability of such compounds as hydroquinone and pyrogallol may be the principal factor in the low toxicity exhibited by these compounds toward *Limnoria*.

The above theory of poisoning assumes, in short, that as soon as a phenol penetrates into tissue cells, pathologic aggregation of the cell proteins is initiated. Of the factors which influence the rate at which this aggregation proceeds, two may be mentioned. These factors, which are themselves conditioned by the special chemical and physical characteristics of the particular phenol, are first, the rate at which the phenol is taken up by tissue lipins; and second, the rate at which the phenol is chemically disintoxicated. When coalescence of the protein particles has reached a stage where redispersion is impossible, the protoplasm may be said to be dead.

The concept of an irreversible aggregation of cellular proteins, as the determinant of death of poisoned cells, immediately raises the question whether protein aggregation may not be the major factor in senescence and in death from so called natural causes. Some preliminary observations with *Limnoria* made by the writer indicate that in poisoning of a severe or fatal grade there is a catalysis of senescence. A similar view was advanced some years ago by Osterhout.[17] The same investigator has pointed out that the resistance of all the cells is permanently lowered by poisoning of any severity.[18] The observations of the writer tend to confirm this view. Further work along this line is in progress.

## SUMMARY.

1. After equilibrium of distribution of a phenol between water and an animal immersed in it has been once attained, the poisoning of the animal proceeds with constant velocity. The criterion of toxicity adopted in the first part of this study was the time required for initial recovery from paralysis after a given time in the phenol solution. In later work observations were made of the percentages of animals which died after stated periods in a phenol solution.

[17] Osterhout, W. J. V., *Science*, 1914, xxxix, 544.

[18] Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Monographs on Experimental Biology, Philadelphia and London, 1922.

2. The numerical value of the velocity constant of poisoning for a given solution is independent of the criterion of toxicity adopted, provided that the criterion serves to measure the intensity of effect of the poison, and not merely the rate at which the poison is absorbed.

3. Recovery from paralysis produced by phenol and death from this poison has the same velocity constant. From this it may be inferred that recovery is due to a reversal of the mechanism which underlies poisoning.

4. The velocity of poisoning by phenols is nearly proportional to the square of the concentration.

5. A strictly chemical (mass law) interpretation is shown to be inadequate for the description of poisoning by phenols.

6. Certain physical factors, involved in poisoning by phenols, are discussed.

7. A precise method for obtaining the velocity constant of poisoning by a given agent is outlined.

# THE RELATION BETWEEN THE ELECTRICAL CONDUCTIVITY OF THE EXTERNAL MEDIUM AND THE RATE OF CELL DIVISION IN SEA URCHIN EGGS.

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(Received for publication, April 3, 1923.)

Our chief purpose in the following experiments has been to determine whether in the case of dividing cells immersed in physiologically indifferent or balanced media of varying salt concentrations there is any definite relation between the rate of cell division and the electrical conductivity (or total ionic concentration) of the medium. For the normal activity of most living cells an essential condition appears to be that the chief ions of the external medium should be present in certain approximately constant (physiologically balanced) proportions. Within a certain range of concentrations the proportions of the salts are apparently of more importance than their absolute concentrations, provided the osmotic equilibrium is not injuriously disturbed; for example, muscle and nerve live almost as long in Ringer's solution diluted with an equal volume (or more) of isotonic sugar solution as in the undiluted medium.[1] In pure sugar solutions these tissues soon lose irritability and transmissivity, but regain these properties on return to Ringer or other appropriate salt solution;[1] these facts show that a certain minimal concentration of salt in the external medium is required for normal activity; the need of a certain minimal electrical conductivity is thus indicated.

The rate of transmission in muscle and nerve in balanced media has recently been found in several instances to vary in a definite manner with the concentration of salts, showing in fact a close paral-

[1] Overton, E., *Arch. ges. Physiol.*, 1902, xcii, 346.

lelism with the electrical conductivity of the medium.[2] This relation is consistent with the view that in these tissues electrical currents traversing the media are an essential factor in transmission.[3] Conceivably the same may be true for other forms of protoplasmic transmission or correlation; thus it might be expected that the rate of such a process as cell division, which apparently also involves transmission (*i.e.*, the coordination of several spatially separate processes), would show a similar dependence in the electrical conductivity of the medium. In the eggs of some marine animals this does not appear to be the case; *e.g.*, the *Fundulus* egg cleaves normally both in sea water and in distilled water.[4] In this case, however, the presence of a water-proof chorion may be a factor—*e.g.*, the egg may be thus insulated from the surrounding medium—and the electrolytes required for the activity of its cleaving portion (blastodisc) may be furnished by the yolk. In other marine eggs, of the small totally cleaving type, *e.g.* sea urchin eggs, cleavage ceases on transfer to isotonic non-electrolyte solutions and is resumed on return to sea water. This parallel with the conditions of activity of muscle and nerve suggests the possibility that a similar correlation between the rate of cleavage and the external conductivity may exist in these eggs, and our experiments have been designed to test this possibility.

The experiments were performed in the summer of 1921 at the Marine Biological Laboratory at Woods Hole. The eggs of the common sea urchin *Arbacia* were used. The eggs were transferred, 10 minutes after fertilization in normal sea water, to mixtures of isotonic sugar solution and sea water, and the intervals between fertilization and cleavage and between first and second cleavages were then determined by direct observation. Standard sugar solutions were prepared (using glass-distilled water) from good commercial preparations of crystallized cane-sugar free from chloride (rock-candy, Domino loaf sugar); the concentration of cane-sugar isotonic

[2] Lillie, R. S., *Am. J. Physiol.*, 1916, xli, 126. Mayer, A. G., *Am. J. Physiol.*, 1915–16, xxxix, 375; 1916–17, xlii, 469; 1917, xliv, 591. Pond, S. E., *J. Gen. Physiol.*, 1920–21, iii, 807.

[3] For a discussion of the part played by electric factors in transmission, *cf.* Lillie, R. S., *Physiol. Rev.*, 1922, ii, 1.

[4] Loeb, J., *Am. J. Physiol.*, 1900, iii, 383.

with the Woods Hole sea water ($\Delta = 1.81°$) is 0.73 M, and this solution was used throughout. The solutions were mixed in glass stoppered graduates and thoroughly aerated. A large volume of solution (250 to 300 cc.) was used in each experiment; the eggs were transferred from the sea water to the solution by pipette, together with a minimal volume of sea water (about 1 cc.), and stirred gently to distribute. Observations were made in watch-glasses under a low power of the microscope.

TABLE I.

*Approximate Time (after Fertilization) in Minutes Required for 50 Per Cent of Arbacia Eggs to Reach 2-Cell and 4-Cell Stages in Normal Sea Water and in Sea Water Diluted with Isotonic Sugar Solution (0.73 M).*

| Experiment No. | Temperature. | Dilution (volumes of sea water in 100 volumes of mixture). | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 100 (control). | 80 | 60 | 50 | 40 | 30 | 20 |
| | °C. | | | | | | | |
| 1 | 22 | 2-cell: 56 | 56 | 56 | | 58 | | 58 |
| | | 4-cell: 83 | 83 | 83 | | 85 | | About 90. |
| 2 | About 21. | 2-cell: 59 | | | 66 | 62 | 62 | |
| | | 4-cell: 95 | | | 105 | 102 | 103 | |
| 3 | 23 | 2-cell: 55 | | | 60 | 60 | 60 | |
| | | 4-cell: 84 | | | 90 | 89 | 85 | |
| 4 | 23 | 2-cell: 54 | | | | 58 | 62 | 62 |
| | | 4-cell: 79 | | | | 83 | 83 | 87 |

The results are best summarized in the form of tables. Table I gives the results of four typical experiments showing the time (in minutes) required for an estimated proportion of 50 per cent of all eggs to reach the 2-cell stage in sea water and in mixtures of sea water and sugar solution ranging between 80 and 20 volumes per cent sea water. The temperature of the solutions in the finger-bowls containing the eggs is given in all cases within half a degree. There is always a variation of some minutes in the time at which different eggs in the same solution begin to cleave, and the cleavage process itself occupies 2 or 3 minutes. These conditions make it somewhat difficult to determine the exact time at which a constant proportion

of eggs have reached a definite stage. The completion of the cleavage furrow was the stage chosen for measurement; from our experience we estimate that the time at which half of the eggs are completely divided, while the rest are still in process of division or undivided, can be determined with a probable error of not more than 2 or 3 minutes. The variability becomes greater as the dilution increases.

It will be seen that as dilution increases the rate of cleavage is little, if at all, affected until the concentration of the salts is reduced to about 30 per cent of the normal. Evidently, therefore, no relation exists between conductivity and rate of cell division within this range of dilutions. With further dilution the rate of cleavage decreases progressively, and more rapidly after 20 per cent dilution is reached than before. Within the range between 20 per cent and 5 per cent dilution decrease of electrolyte content is quite definitely associated with decrease in rate of cleavage, but no constant proportionality between the two is indicated by our experiments. In mixtures containing 10 per cent sea water (Table II) the rate of cleavage is approximately from three-quarters to two-thirds that in normal sea water, and in 6 per cent sea water the rate is reduced to about one-half the normal. Many eggs fail to cleave when this dilution is reached and the proportion of non-cleaving eggs increases rapidly with further dilution. In 2 per cent sea water and in pure sugar solution the eggs never cleave, although they remain living for some hours and will resume cleavage if returned to sea water.

Table II summarizes the results of eight typical experiments with the lower concentrations of sea water (10 to 2 volumes per cent), carried out between July 19 and July 29, 1921. The eggs were normal and the range of temperature was 21–24°C. Six other similar experiments not cited in the table yielded results of the same general character, but the rate of cleavage was somewhat slower than normal and in three experiments the temperature was below 20°C. The eight experiments cited are those in which the conditions and the controls were most nearly uniform. Pure sugar solution and 2 per cent sea water were also used in all experiments, but no cleavage occurred in these solutions.

This region of dilution (from 20 to 4 volumes per cent sea water) shows most distinctly the effect of varying the concentration of salts. In the more dilute solutions, from 6 volumes per cent down, there arises the difficulty of a greatly increased variability in the cleavage rates of different eggs; many eggs fail entirely to cleave, or the cleavage is so retarded that the exact time ceases to have significance. In this series we found it more satisfactory to measure the time required for 25 per cent (instead of 50 per cent) of the eggs to reach

TABLE II.

*Minutes between Fertilization and Completion of First Cleavage.*

| Experiment No. | Temperature. | Dilution (volumes of sea water in 100 volumes of mixture). | | | | | |
|---|---|---|---|---|---|---|---|
| | | 100 (control). | 10 | 8 | 6 | 5 | 4 |
| | °C. | | | | | | |
| 1 | 24 | 51 | 74 | 83 | 100 | | 20 per cent cleaved after 180 min. |
| 2 | 24 | 54 | 63 | 85 | 93 | | 10 per cent cleaved after 145 min. |
| 3 | 21 | 54 | 75 | 94 | 108 | | 10 per cent cleaved after 150 min. |
| 4 | 21 | 56 | 79 | 90 | 107 | 12 per cent cleaved after 150 min. | 5 per cent cleaved after 150 min. |
| 5 | 21 | 59 | 89 | 105 | 16 per cent cleaved after 160 min. | 1 per cent cleaved after 165 min. | 0.3 per cent cleaved after 165 min. |
| 6 | 22.5 | 52 | 85 | 95 | 106 | 114 | 5 per cent cleaved after 165 min. |
| 7 | 23 | 50 | 82 | 125 | About 3 per cent cleaved after 170 min. | About 3 per cent cleaved after 170 min. | 1 per cent cleaved after 170 min. |
| 8 | 23 | 50 | | 109 | 10 per cent cleaved after 170 min. | 8 per cent cleaved after 173 min. | 1 per cent cleaved after 175 min. |

the 2-cell stage. At dilutions of 4 and 5 per cent, relatively few eggs (usually from 5 to 10 per cent) cleave within 2 or 2½ hours; and at a dilution of 2 per cent cleavage fails entirely. Apparently the lowest concentration of electrolytes at which cleavage is possible is one corresponding to about 4 per cent sea water. Below this concentration the rate of some of the processes concerned in cell division appears to be decreased to such a degree that cleavage is unable to

complete itself. There is evidence that the cytoplasmic rather than the nuclear processes are primarily affected by deficiency of electrolytes in the medium.[5]

Although the eggs remain uncleaved in the more dilute solutions and in pure sugar solution, they remain living for at least several hours and capable of resuming cell division and development when returned to sea water. We have not made a detailed study of the maximum length of life in the different solutions, but in all of the experiments of Table II part of the eggs were returned to sea water after 2 to 2½ hours, and the proportion developing to a blastula stage was determined. In the less dilute solutions (6 volumes per cent and stronger) the eggs are only slightly affected by this exposure and the great majority form active blastulæ. In solutions containing 4 per cent sea water, 50 per cent of the eggs formed blastulæ in four out of the eight experiments of Table II after exposures varying from 1¾ to 2½ hours, and a varying number reached this stage in the other four experiments. In 2 per cent sea water these proportions were not greatly altered.[6] In the pure sugar solution the rate of deterioration was decidedly more rapid; yet in all but one of the eight experiments a certain proportion of blastulæ (in most cases less than 5 per cent) developed from eggs which had remained for 2¼ to 2½ hours in the solutions. In two other experiments in which eggs were transferred to sea water after 98 and 110 minutes (respectively) in the sugar solution, 50 per cent or more formed blastulæ. Allowance has to be made for the small quantity of sea water introduced with the eggs; the experiments with 2 per cent sea water indicate that a slight trace of salt may have a well marked protective influence.

The curve (Fig. 1) shows graphically the general manner in which the rate of division varies with the concentration of salts. Ordi-

[5] Some years ago (Lillie, R. S., *Biol. Bull.*, 1902–03, iv, 164), I observed in *Arbacia* eggs nuclear division without cytoplasmic division in solutions of non-electrolytes.

[6] Loeb (Loeb, J., *Biochem. Z.*, 1910, xxix, 80) found that in a mixture of 49 volumes of 0.75 M dextrose *plus* 1 volume of sea water the great majority of *Arbacia* eggs died in 3 hours if oxygen was present. In absence of oxygen or presence of cyanide life was greatly prolonged.

nates are volumes per cent of sea water in the mixtures, abscissæ the observed intervals between fertilization and the formation of the first cleavage furrow. The points are averages of the observations in the tables; these observations are few, but sufficient to show the general course of the curve; the horizontal direction toward its lower end indicates absence of cleavage in concentrations below 4 per cent.

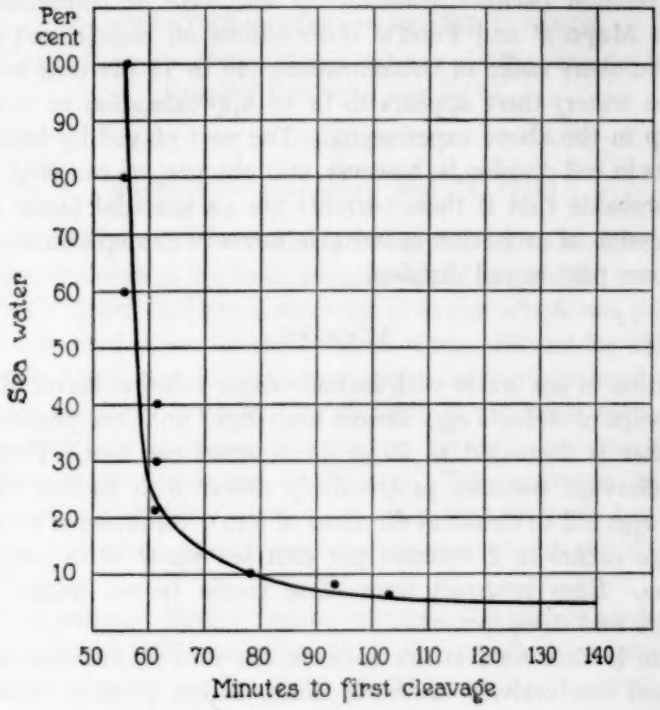


FIG. 1.

We conclude that under normal conditions the rate of the cell division process is determined by factors which are independent of the electrical conductivity of the external medium within a wide range of variation. Conductivity may be reduced to less than one-third of the normal without significantly altering the rate of division. Our experiments suggest, however, that in media of lower salt content electrical conductivity may become a limiting factor; *i.e.*, the

rate possible to the entire division process may in these solutions be controlled by the conductivity. It seems probable that for the other factors to be effective a certain minimal external conductivity is required; when the conductivity falls below this critical value a correlation between the conductivity and the rate of the entire composite process of cell division may then appear. An apparently linear relation between conductivity and rate of transmission is seen in Mayer's[2] and Pond's[2] observations on muscle and nerve. Within a short range of concentrations (10 or 15 per cent to 5 per cent sea water) there appears to be an approximation to this relationship in the above experiments. The part played by bioelectric currents in cell division is, however, still obscure; we can only regard it as probable that if these currents are an essential factor in the transmission of excitation in irritable forms of protoplasm they also play some part in cell division.

## SUMMARY.

Dilution of sea water with isotonic sugar solution leaves the rate of cleavage of *Arbacia* eggs almost unchanged until the proportion of sea water is decreased to 20 or 25 volumes per cent. From this point cleavage becomes progressively slower with further dilution. Many eggs fail to cleave at dilutions of 5 to 6 volumes per cent. No cleavage occurs in 2 volumes per cent sea water or in pure sugar solution. Eggs returned from these media to sea water resume cleavage and development.

There is thus no relation between the rate of cleavage and the electrical conductivity of the medium, except possibly within the range of dilutions from 20 to 5 volumes per cent sea water. In this range cleavage rate decreases as conductivity decreases, but the relation is not a linear one.

# A DISSECTION OF THE CHROMOSOMES IN THE POLLEN MOTHER CELLS OF TRADESCANTIA VIRGINICA L.

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PLATE 1.

(Received for publication, May 21, 1923.)

The micro dissection method is proving of great value for studying the physical nature of the fresh, unstained, and living cell.

Barber's pipette holder,[1] a mechanical device which was primarily intended by its designer for the isolation of bacteria and for which it is admirably fitted, has hitherto been quite successfully used for the dissection of cells. With it Kite and one of us[2] have been able to make preliminary studies on the nuclear network and even to drag individual chromosomes out of the cell. The procedure, however, was exceedingly difficult owing to the fact that Barber's instrument lacks the necessary control over the movements of the dissecting needles.

With a recently devised micro manipulator[3] which works on a principle entirely different from that of Barber's instrument, the movements of micro needles under the highest magnifications of the microscope can be controlled to such an extent as to render possible an actual dissection of the chromosomes of a cell. A description of such operative work on the germ cells of certain insects is shortly to be published.

For the dissections recorded in this paper a type of micro needle was used which has not yet been described. The needle is made of glass in the usual way except that during the moment when its tip is being

[1] Barber, M. A., *Philippine J. Sc.*, 1914, ix, 307.

[2] Kite, G. L., and Chambers, R., *Science*, 1912, xxxvi, 639. Chambers, R. *Science*, 1914, xl, 824; 1915, xli, 290.

[3] Chambers, R., *Anat. Rec.*, 1922–23, xxiv, 1.

drawn in the micro burner, a peculiar twist is given which causes the finely tapering tip of the needle to bend sharply at about 20 to 30 microns from its apex. This gives one a remarkably fine hooked needle with a very slender tapering tip set on a relatively stout shank to ensure adequate rigidity.

The pollen mother cells of *Tradescantia* were selected, owing to the ease with which the desired stages can be obtained and because of the conspicuous chromosomes which are present during the mitotic stages. The fully grown cell is more or less spherical and about 65 microns in diameter. An anther of young flower buds was crushed and the pollen mother cells set free on a cover-slip in a drop of equal parts of 10 per cent saccharose and of sap expressed from a freshly cut end of the *Tradescantia* stalk. The drop was spread out in a thin layer on a cover-slip which was then inverted over a Barber moist chamber on the stage of the microscope. The micro needles which were used for the dissection projected into the chamber.

All the observations and experiments recorded were made with a Zeiss 2 mm. apochromatic objective, N. A. 1. 40, and a No. 8 compensating ocular. It is worthy of note that the photographs illustrating the experiment were taken with the microscope set up for micro dissection purposes which necessitated a substage condenser with a free working distance of $\frac{3}{8}$ inch. This distance was secured by using a Leitz substage aplanatic condenser with the top lens removed. A layer of immersion oil was placed between the condenser and the floor of the chamber, so that the breaking of the light rays occurred only as they passed into the space of the chamber to reach the hanging drop containing the cells. The combination of lenses used with this arrangement and the illuminant passing through a B filter resolves the structure of the *Surirella gemma* shell but not that of the *Amphipleura pellucida*.

Fig. 1 is a photograph of living, unstained pollen mother cells as the chromosomes are collecting in the equator prior to the first maturation division. Note the ring shape of two of the chromosomes in one of the cells. The chromosomes lie in an optically structureless or hyaline area, which occupies a large part of the cell. About this hyaline area is a relatively thin zone of cytoplasm in which occur highly refractive globules apparently of a fatty nature. In killed and stained cells this hyaline area exhibits fibrous streaks, the so called

spindle fibers of the metaphase. The cell is enclosed within a thin cellulose wall which is somewhat out of focus in this figure.

In the attempt to dissect the cells, it was found necessary to remove the investing cellulose wall as it is too stiff to allow the ready penetration of the micro needle.

Fig. 2 shows the way in which this is done. The cellulose wall is first punctured and torn with a needle. The needle is then thrust through the gap and inserted into the viscous cytoplasm of the cell. With a second needle the opposite side of the cellulose wall is caught, and, on moving this needle while the first is kept stationary, the cellulose wall is pulled cleanly off the cell. In the early prophase stages of the cell this was not possible as the wall was found to be much less developed and was closely adherent to the underlying cytoplasm. When the cell reaches the metaphase stage, the investing wall has become much stiffer and is separable from the cytoplasm.

Fig. 3 shows the cellulose wall after it has been removed and the naked cell lying beside it. The chromosomes are somewhat out of focus but they can still be seen in the hyaline area.

That this hyaline area is jelly-like in consistency is shown in Fig. 4, in which it has been stretched by inserting two needles and then moving them apart. Chromosomes which lie in the stretched part of the area tend also to be stretched. On releasing one needle the stretched area gradually tends to revert to its original shape.

Fig. 5 shows that the cytoplasm can be torn away from the hyaline nuclear jelly in the form of glutinous, granular strips.

Fig. 6 follows Fig. 3 in demonstrating the removal of chromosomes out of the jellied area into which a vertical needle has been thrust. The tip of this needle is out of focus and its hollow shaft shows in the photograph in cross-section as a ring. The other needle with its tip also out of focus lies diagonally to the plane of the figure and is in position preparatory to removing one of the chromosomes.

In fresh preparations of pollen mother cells it is difficult to pull the chromosomes out of the jelly. The chromosomes are considerably more solid than the material in which they lie, but in the attempt to extricate one, they all tend to clump and to stick together. After some time, however, the hyaline area tends to liquify and it is then easier to isolate the chromosomes.

Fig. 7 shows two isolated chromosomes. Since these chromosomes are from a cell which has not yet undergone the first maturation division, they are morphologically tetravalent. One of them is a definite ring with transverse constrictions. The other is almost a closed V with one part on end viewed in optical section. It is distinctly a cylinder exhibiting a medulla, which possesses a refractive index quite different from that of its cortex. This structure is in accordance with that of the prophase filaments already reported and substantiates the findings recorded in a discussion on the structure of the chromosome, by one of us.[4]

Fig. 8 shows one half of the tetravalent ring-shaped chromosome the other half having been dissected away. Notice the tip of the dissecting needle at the lower left corner of the figure.

Fig. 9 exhibits the elasticity of the chromosome material. The chromosome of the previous figure was caught at its two ends by the needles and stretched. It is to be noted that the constricted area stretches more than the rest of the rod and is thus drawn out into a thin filament. Stretching the chromosome slightly more than is shown in the figure causes a break at the constriction, whereupon the slender filamentous portion gradually draws back into the more massive body of the chromosome.

Regarding the structure and consistency of the metaphase spindle and of the chromosomes of these cells, we can draw the following conclusions.

The spindle area is a hyaline, jelly-like mass, less solid than the surrounding cytoplasm and distinctly separated from it. In the living and fresh state there is no evidence during metaphase of spindle fibers which form so prominent a feature in coagulated and stained material.

In this jelly lie the much denser but homogeneous chromosomes. The chromosome of the *Tradescantia* pollen mother cell is an elastic, jelly-like, nodulated, cylinder and possesses a cortex which differs markedly in refractive index from its central core.

[4] Sands, H. C., *Am. J. Bot.*, 1923, x (in press).

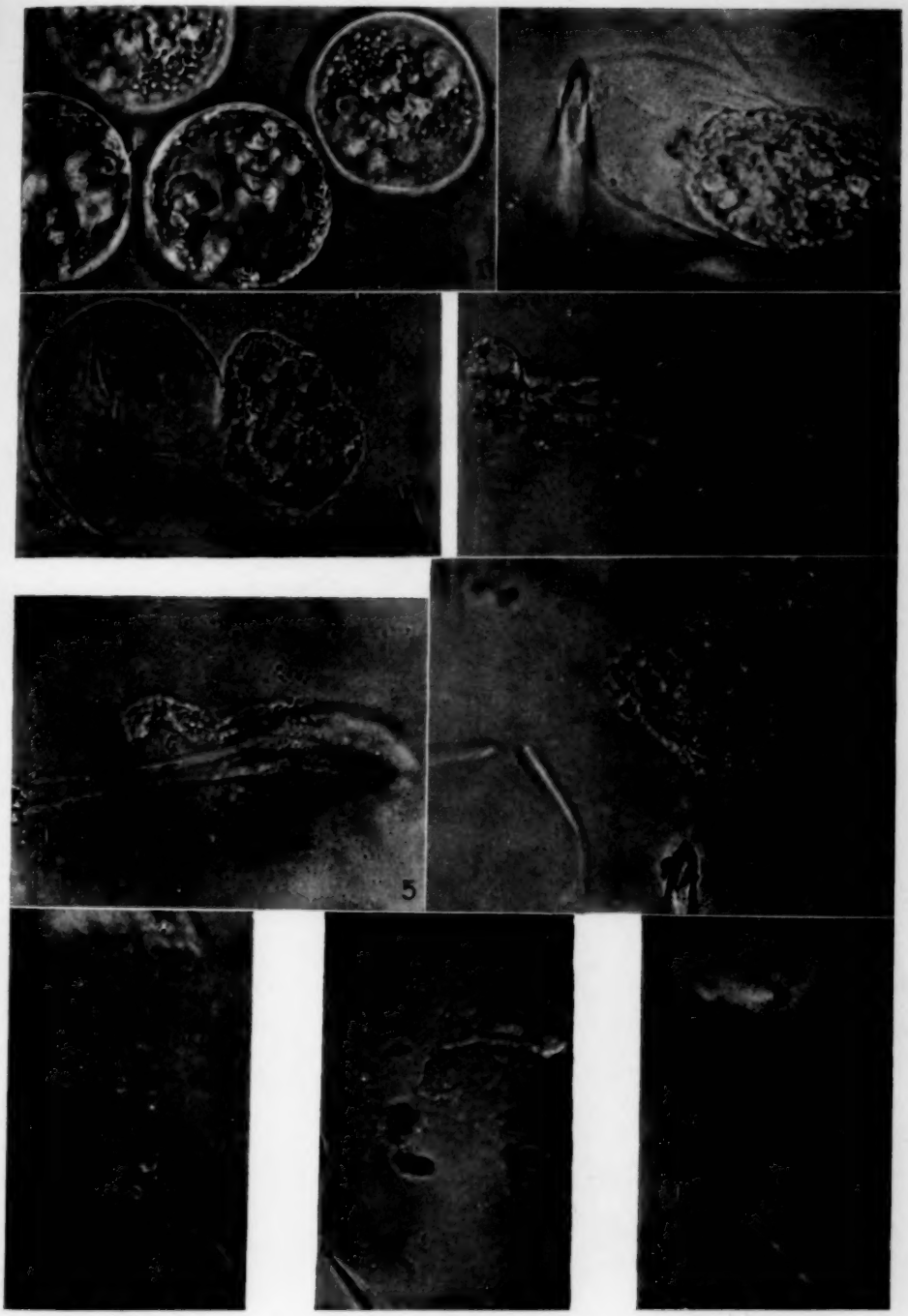
EXPLANATION OF PLATE 1.

FIGS. 1 to 9. Photomicrographs of living unstained pollen mother cell of *Tradescantia virginica* L. isolated in plant sap and saccharose solution and dissected to ascertain the consistency of the nuclear area and of the chromosomes.

Photographs taken with a No. 8 compensating ocular and a Zeiss 2 mm. apochromatic objective. Substage equipped with a Leitz aplanatic condensor with the top lens removed. Source of illumination a Leitz Liliput arc lamp with the light rays passing through a B filter. This combination resolves the beaded lines on the shell of *Surirella gemma* but not those of *Amphipleura pellucida*.

The photographs were reduced to about $\frac{1}{1}\frac{3}{8}$ of the original size.

For a description of the figures see the text.




(Chambers and Sands: *Tradescantia virginica* L.)

# THE MECHANISM OF THE ENTRANCE OF SPERM INTO THE STARFISH EGG.*

By ROBERT CHAMBERS.

*(From the Cornell University Medical College, New York.)*

(Received for publication, May 24, 1923.)

The fertilization process in echinoderm eggs has been the subject of a great deal of investigation. It is very striking, however, to find so few actual observations on the manner in which the spermatozoon reaches and enters the egg. This may be accounted for by the fact that most workers have relied upon the early researches of Hermann Fol,[1] who first observed the entrance of a spermatozoon into an animal egg. Fol described the process in such detail that even recent text-books comment upon the completeness of his observations. From his researches on the starfish egg, Fol concluded that the presence of spermatozoa in the immediate vicinity causes the egg to respond by forming on its surface a conical elevation which attracts the spermatozoon from a distance. This cone he called the attraction cone. This rather extraordinary interpretation has been generally accepted to account for the arrival of the spermatozoon on the surface of the egg. Among the few who have taken exception to this view is Buller,[2] who claimed that the egg exerts no directive action and that the spermatozoa accumulate in the jelly surrounding the egg because of their positive stereotropism.

Buller studied only those species whose eggs possess a jelly readily penetrable by its own spermatozoa. The starfish spermatozoa have very blunt heads and are apparently unable to penetrate the very adhesive jelly which surrounds the starfish eggs. They come into

* The experiments, upon which this paper is based, were conducted in the Research Division of Eli Lilly and Company, at the Marine Biological Laboratory, Woods Hole.

[1] Fol, H., *Compt. rend. Acad.*, 1876, lxxxiii, 667; *Mem. Soc. phys. et hist. nat., Genève*, 1879, xxvi, 89.

[2] Buller, A. H. R., *Quart. J. Micr. Sc.*, 1902, xlvi, N. S., 145.

contact with the outer border of the jelly, in which they appear to become inextricably entangled. Fol himself commented upon this and tried to explain the passage through the jelly of the spermatozoa which succeed in fertilizing the egg, by assuming that the cone which forms on the egg's surface at this time exerts an attraction which in some mysterious way draws the spermatozoon through the jelly to it.

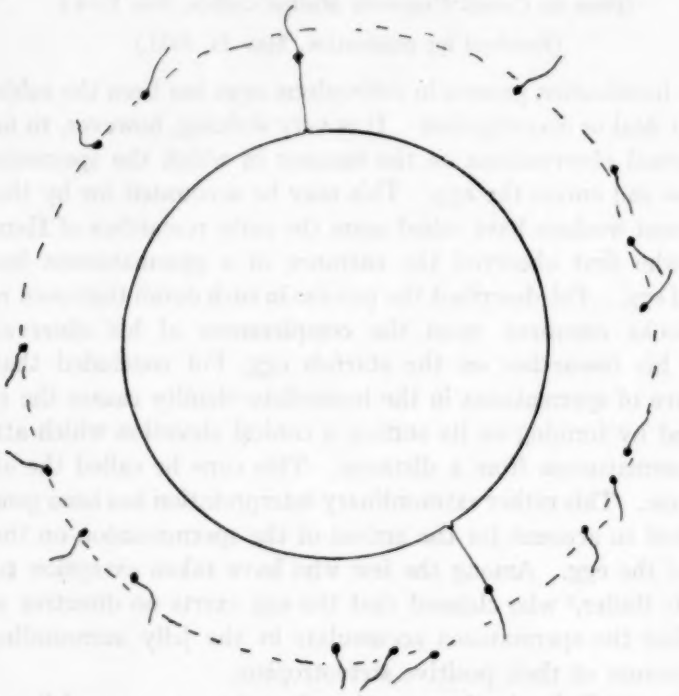


FIG. 1. Diagram of a starfish egg 1 minute after insemination. Spermatozoa are caught in the outer border of the jelly indicated here in outline. Two spermatozoa are advancing through the jelly to the egg and from each extends a visible line to the summit of a cone on the egg's surface.

The results recorded in this paper constitute an attempt to explain the peculiar behavior of the starfish spermatozoon which enables it to migrate through the jelly of the egg.

When an egg is observed within thirty seconds of its insemination, there may be seen, among the many spermatozoa which adhere to the

outer surface of the jelly, one or several spermatozoa whose heads are advancing steadily into the jelly towards the egg (Fig. 1). During its course the head does not deviate from a straight line except when it occasionally gives a spasmodic twitch. Its tail usually trails motionless behind but may sometimes lash feebly to and fro. On allowing one's eye to travel ahead of the spermatozoon to the egg, one will see a

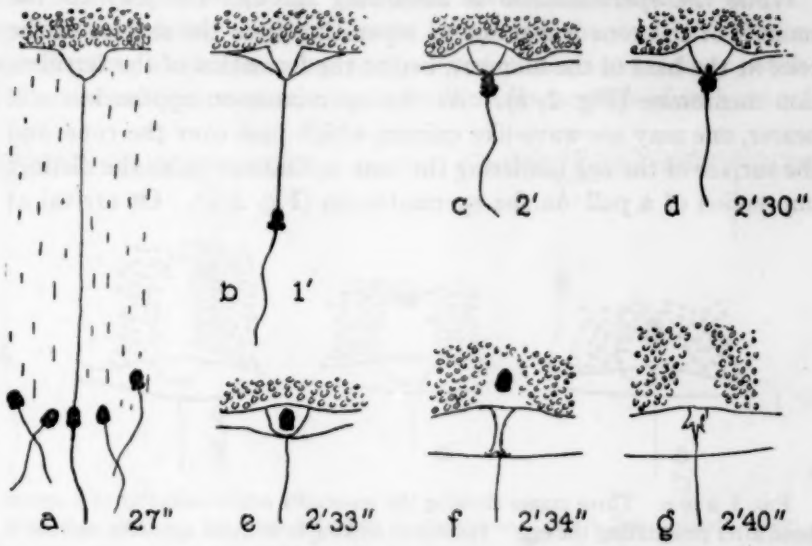



FIG. 2, *a* to *g*. Seven steps in the passage of a spermatozoon through the jelly and into the living egg. The whole process figured here lasts about 2½ minutes. (*a*) 27 seconds after insemination. From the fertilization cone extends a delicate filament to the periphery of the jelly where the tip of the filament has touched a sperm head. (*b*) 30 seconds later. The spermatozoon has been dragged halfway through the jelly. At the base of the cone the fertilization membrane is rising. (*c*) 1 minute later. The sperm head has reached the cone. (*d*) The tip of the sperm head is narrowing as it passes through the fertilization membrane into the cone. (*e*) The head is within the cone. (*f* and *g*) The sperm head is sinking into the egg and the fertilization cone is being replaced by what Fol termed the exudation cone.

conical elevation on the egg, from the summit of which a tenuous but distinctly visible line extends to the head of the advancing spermatozoon. Owing possibly to the extreme tenuity of this line, I have been unable to observe the filament itself except when the cone of the egg

on the one hand and the head of the spermatozoon on the other can be utilized as a guide. The earliest stage at which it has been observed is shown in Fig. 2, *a*, in which it reaches the periphery of the jelly. Evidence is given below to show that this line is a filamentous process which grows out from the summit of a conical elevation on the egg through the intervening jelly.

While the spermatozoon is advancing through the jelly to the summit of the cone a membrane, separating from the surface of the cone at the base of the filament, begins the formation of the fertilization membrane (Fig. 2, *b*). As the spermatozoon approaches still nearer, one may see wave-like quivers which pass over the cone and the surface of the egg bordering the cone so that one gains the distinct impression of a pull on the spermatozoon (Fig. 2, *c*). On arrival at

FIG. 3, *a* to *c*. Three stages showing the unusually active behavior of a sperm head after penetrating the egg. Instead of sinking in without apparent motion it wriggled from side to side for one minute and finally worked its way among the cytoplasmic granules to one side of the hyaline pathway.

the summit of the cone there ensues a pause of about thirty seconds during which time the sperm head occasionally gives one or two convulsive twitches. The sperm head then narrows at its tip (Fig. 2, *d*), and lengthens out (*e*), as it slips rapidly through the fertilization membrane to round out again after it has entered the substance of the cone (Fig. 2, *f*). There apparently exists a pore in the fertilization membrane through which the filament previously extended and which is now the only means of ingress for the spermatozoon. The cytoplasmic granules at the base of the cone disappear in front of the spermatozoon to form a hyaline pathway along which it glides into the egg. Occasionally, a sperm head, after having entered the egg (Fig. 3, *a* to *c*), keeps on oscillating from side to side and finally

leaves the pathway prepared for it and crowds in among the closely packed cytoplasmic granules where it gives rise to a typical sperm aster quite out of the usual position.

The egg does not necessarily form one fertilization cone only. If over inseminated it may form several cones at different spots on its surface. Each filamentous process of the cones may have a spermatozoon attached to its tip. We then have the spectacle of several spermatozoa being steadily drawn in toward the egg. If all the spermatozoa start advancing at the same instant and if they reach the surface of their respective cones within a few seconds of each other,

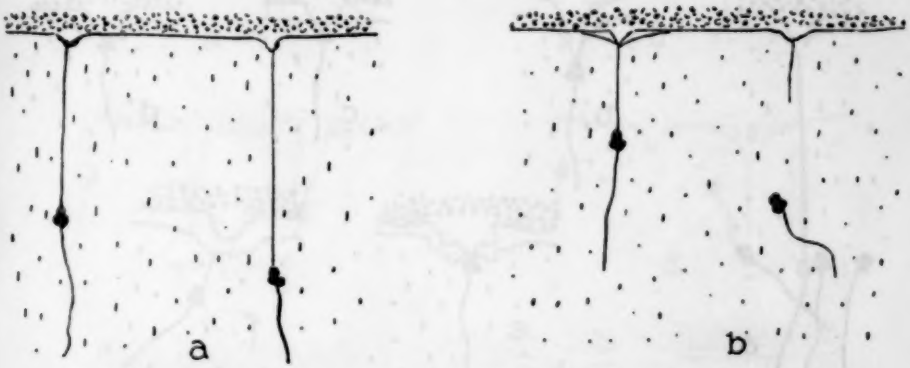



FIG. 4, *a* and *b*. (*a*) Two attached spermatozoa. (*b*) A few seconds later; the more advanced one is pursuing its course while the other has been released and lies motionless in the jelly.

they all pass successfully into the egg. This, however, does not usually occur in healthy mature eggs. One of the cones tends to form before the others and the spermatozoa seldom travel through the jelly at the same rate of speed. Before they are more than half way through the jelly the filaments which are behind hand often lose their spermatozoa (Fig. 4). The released spermatozoa remain inextricably entangled in the jelly while the naked filaments withdraw and their cones sink back into the egg. This reaction is presumably a response emanating from the filament which first came into contact with a spermatozoon.

Another phenomenon also occurs to protect the egg from polyspermy. Beginning at the cone which was the first to secure

a spermatozoon, the egg membrane rises to form the well known fertilization membrane. The rising of this membrane spreads rapidly over the egg. Fig. 5 shows a case in which a delayed filament did not lose attachment to its sperm. The spermatozoon was steadily drawn in until it reached the summit of the cone. By this time, however, the fertilization membrane had spread over the region and proved to serve as a barrier to the entrance of the spermatozoon. The cone spread out at its base and formed accessory elevations on its surface. The fertilization membrane also wrinkled con-

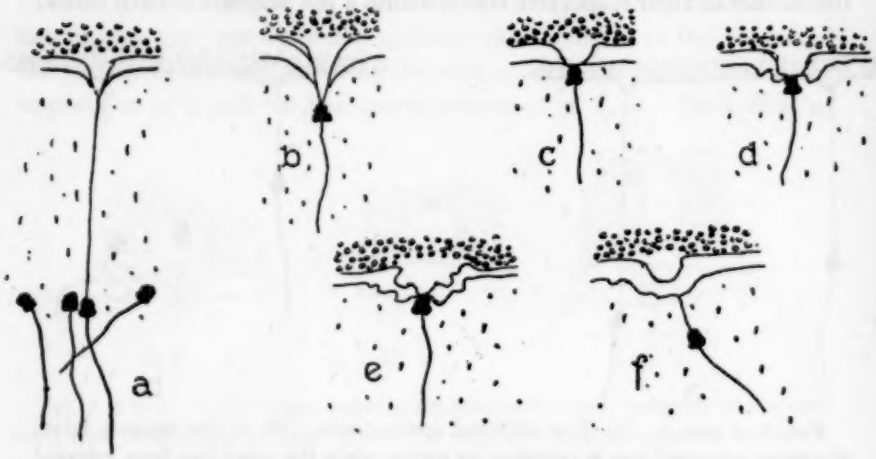



FIG. 5, *a* to *f*. Six stages in the advance of a spermatozoon towards the cone of an egg which has already formed a fertilization membrane from another source. Note in (*d*) and (*e*) the wrinkling of the fertilization membrane and the irregular elevations which form on the cone of the egg. In (*f*) the cone has definitely receded from the fertilization membrane leaving the spermatozoon outside.

siderably but when the cone finally withdrew from the membrane, the spermatozoon was still outside. Such a spermatozoon usually springs away for a short distance in the jelly where it remains permanently motionless and attached to the membrane by a slender thread. The protective action of this membrane was first shown by Fol.

Eggs aged by standing in sea water for several hours lose their protective reaction against polyspermy. In the presence of spermatozoa the surface of an old egg forms numerous cones and the filamentous process from each one quickly secures a spermatozoon. None of the

filaments tend to lose their spermatozoa, which are usually readily taken in, even after the fertilization membrane has been formed.

We have thus far described the formation of cones, all of which happen to lie in direct alignment with particular spermatozoa. This may indicate that the spermatozoon is directly responsible for the

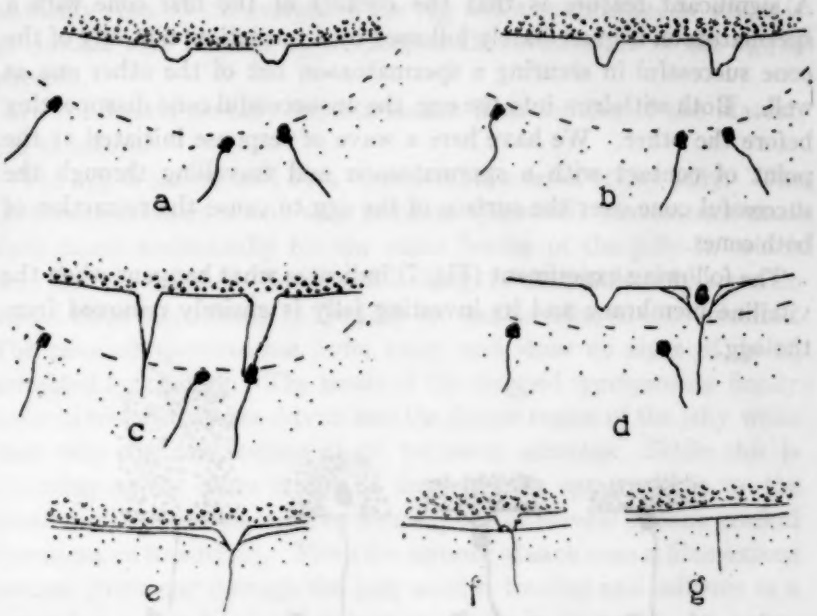



FIG. 6, *a* to *g*. Seven stages in the insemination of an egg from which the investing jelly was pulled away at one place on its surface. (*a*) Two cones have formed on the bared surface of the egg. (*b*) Both cones are growing through the artificial space. (*c*) One of the cones has come into contact with a spermatozoon which lies motionless in the jelly. The other cone has extended a filamentous process into the jelly but has met with no sperm. (*d*) Both cones are receding, one of which has secured a spermatozoon. (*e*) The unsuccessful cone has disappeared. (*f*) The successful cone has been replaced by Fol's exudation cone. (*g*) This also has disappeared in its turn. The gradual obliteration of the artificial space is due possibly to a rounding of the egg upon fertilization.

conical elevation on the surface of the egg just opposite it. That this is not necessarily the case is shown in Fig. 6. Here the jelly was pulled away from the egg at one spot by means of micro dissection needles, in order to see whether a cone would develop a filamentous

process if the jelly be absent. Two cones happened to form in the denuded area. In Fig. 6, *a* and *b*, we see both cones growing out into the space between the egg and the jelly. In *c* they have reached the jelly into which they now extend as filamentous processes. One cone came into contact with a spermatozoon. The other did not. A significant feature is that the contact of the first cone with a spermatozoon is immediately followed by a retraction, not only of the cone successful in securing a spermatozoon but of the other one as well. Both withdrew into the egg, the unsuccessful cone disappearing before the other. We have here a wave of response initiated at the point of contact with a spermatozoon and travelling through the successful cone over the surface of the egg to cause the retraction of both cones.

The following experiment (Fig. 7) indicates what happens when the vitelline membrane and its investing jelly is entirely removed from the egg.

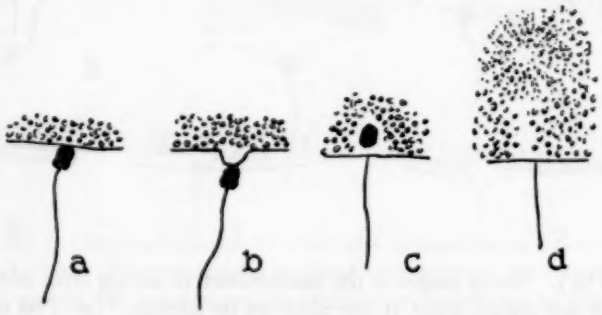



FIG. 7, *a* to *d*. Four stages in the insemination of an egg which was stripped not only of its jelly but also of its membrane. (*a*) A spermatozoon is touching the surface of a completely naked egg. (*b*) A blister-like elevation has formed where the spermatozoon was in contact with the egg. (*c*) The spermatozoon has sunk into the egg. Note the absence of a fertilization membrane. (*d*) 7 minutes after insemination the sperm head has begun to form an amphiaster.

By careful manipulation with the micro dissection instrument an egg was successfully shelled out of its membrane (Fig. 7). The egg rendered naked in this way was inseminated. When a spermatozoon touched its surface (Fig. 7, *a* to *d*) a blister-like elevation quickly formed into which the sperm head was immersed. No fertilization membrane developed. In spite of this the egg segmented in the normal manner, Fig. 7, *d* showing the sperm aster.

The formation of the cone on the surface of the starfish egg is not necessarily a response of the egg to any spermatozoon which happens to come into contact with it. I have frequently observed spermatozoa collide with the surface of unfertilized, naked eggs without causing the egg to respond in any way. After a few seconds, however, a papilla suddenly rises on the surface of the egg and a spermatozoon which is in contact with it sinks in, whereupon the fertilization of the egg is effected.

In conclusion we may summarize the insemination of the starfish egg as follows:

The egg is surrounded by a considerable zone of sticky jelly. The spermatozoa swim about with peculiar spasmodic movements until their heads accidentally hit the outer border of the jelly in which they become caught. Continued lashings of their tails only serve to imbed them more firmly in the jelly or, occasionally, to set some free. The released spermatozoa swim away and show no signs of being attracted by the egg. The heads of the trapped spermatozoa finally come to rest as they are driven into the denser region of the jelly while their tails continue lashing about for many minutes. While this is occurring on the outer border of the jelly, the egg responds to the presence of the spermatozoa by forming one or several hyaline conical elevations on its surface. From the summit of each cone a filamentous process grows out through the jelly until it touches and adheres to a motionless sperm head which happens to lie in its path. An extraordinary reaction now takes place. The filament immediately begins to retract, dragging the spermatozoon with it through the jelly to the summit of the cone on the surface of the egg. At the same instant all the other filaments are similarly withdrawn whether they have each secured a spermatozoon or not. The fertilization membrane rises and spreads from the region of the cone whose filament first made contact with a spermatozoon. When the spermatozoon arrives on the cone it is engulfed by the cone and then passes deeper into the egg, where it gives rise to the amphiaster of the fertilized egg.[3]

[3] Further details of this study will be published in the near future.

# THEORY OF REGENERATION BASED ON MASS ACTION.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 15, 1923.)

## I.

When a living organism is mutilated, some new growth will generally ensue which would not have occurred without mutilation. Such growth is designated as regeneration for the reason that not infrequently the new growth results in an approximate restoration of the old form of the mutilated organism. Two problems arise; namely, first, to correlate the mutilation with the new growth, and second, to explain the fact that the new growth tends in certain cases towards the restoration of the old form. The two problems are by no means identical though they are often treated as if they were so. Among the older suggestions concerning such a correlation, only that made by Sachs[1] seems of value; namely, that as a consequence of the mutilation, the flow of material in a plant is blocked in the neighborhood of the wound and that this leads to a new growth in the place where the block occurs. This is only part of Sachs' suggestion, the other part being that this material is not the carbohydrates, proteins, or fats, which form the bulk of the living organism, but specific organ-forming substances which he assumed to be as numerous as the morphological differences in the organs; and this was supposed to explain why the new growth tends to restore the old form. These hypothetical specific organ-forming substances, which are assumed to exist in only minute quantities, would today, perhaps, be called hormones. Since a scientific theory must rest on quantitative experiments, which are out of the question as long as the quantities to be measured are as hypothetical as are the specific organ-forming substances of Sachs, this part of his idea has led to no progress. If, however, we disregard

[1] Sachs, J., Stoff und Form der Pflanzenorgane, *Arbeiten des botanischen Instituts in Würzburg, 1878–82*, Leipsic, 1882, ii, 452.

the idea of specific organ-forming substances for the present, focusing our attention on the relation between the measurable quantity of dry matter of the old plant and the quantity of dry matter regenerated in a given time, the law of mass action makes it possible to correlate the mutilation with the process of regeneration. Some of the experiments on which this conclusion is based have already been published[2] and it is intended in this paper to make the proof more complete.

It has long been known that a leaf of *Bryophyllum calycinum* forms new roots and shoots in its notches when it is detached from the plant and kept in moist air or on moist soil. When we determine the dry weight of the roots and shoots produced by such leaves in a given time, it can be shown that they are approximately in direct proportion to the dry weight of the leaves themselves. In order to furnish such a proof, the amount of growth or regeneration must be compared in sister leaves; *i.e.*, in leaves which come from the same node of the same plant. In each node of *Bryophyllum* there are two leaves which have the same age and, as a rule, the same history, and only such leaves are strictly comparable in regard to quantity and efficiency of chlorophyll. We make the assumption that sister leaves, when exposed to equal conditions of illumination, moisture, temperature, and chemical environment will produce approximately equal quantities of products of assimilation in equal times, per gram of dry matter of leaf. This assumption seems to be justifiable on the basis of our present knowledge.

What we can actually prove and have proved already[2] is that equal masses of dry weight of sister leaves, when detached from the plant, produce in equal times under equal conditions of illumination, moisture, temperature, and chemical environment approximately equal masses of dry weight of roots and shoots from their notches. For the convenience of the readers some new experiments confirming the old results may be described.

*Experiment 1.*—Seven pairs of sister leaves of equal size were detached from stems and were suspended in an aquarium so that their apices dipped in water (Fig. 1). The experiment lasted from March 20 to April 12, 1923. From the notches near or in the water new roots and shoots arose. It was expected that each leaf of a

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 297, 651.

pair would produce the same mass of roots and shoots from the notches, or that the seven leaves, one each from a pair, would produce the same mass of roots and shoots as their seven sister leaves, per gram dry weight of leaves. This was found to be approximately correct. The roots and shoots were removed from the leaves at the end of the experiment and leaves, roots, and shoots were dried for 24 hours in an electric oven at about 100°C. The two sets of sister leaves are designated as Set I and Set II in Table I.

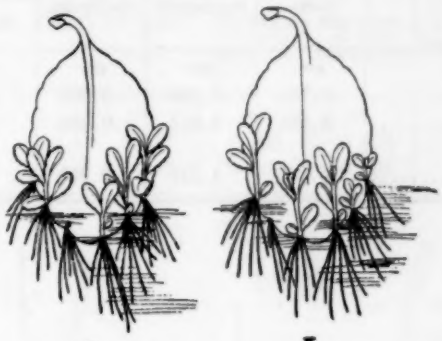



FIG. 1. Isolated sister leaves dipping with their apices into water. March 20 to April 11, 1923. Equal masses of leaves producing equal masses of shoots and roots.

TABLE I.

| | Dry weight of leaves. | Dry weight of shoots regenerated. | Dry weight of roots regenerated. | 1 gm. dry weight of leaves produced. | |
|---|---|---|---|---|---|
| | | | | Shoots. | Roots. |
| | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* |
| Set I | 1.528 | 0.405 | 0.153 | 265 | 100 |
| " II | 1.665 | 0.464 | 0.166 | 278 | 100 |

Each of the two sets of seven leaves produced therefore approximately equal masses of dry weight of shoots and roots per gram dry weight of leaves.

*Experiment 2.*—Nineteen pairs of sister leaves were used, one leaf of each pair was left intact, while each sister leaf was cut into a small apical and a larger basal piece. All dipped with the apical end into water (Fig. 2). The figure shows that the roots and shoots produced by sister leaves varied approximately in pro-

portion to the mass of the pieces of the leaves. This was confirmed by the measurements in Table II. The experiment lasted from March 26 to April 17, 1923. The nineteen whole leaves are termed Set 2, the nineteen sister leaves each cut into two pieces, 1*a* and 1*b*, are termed Set 1.

TABLE II.

| | Dry weight of leaves. | Dry weight of shoots regenerated. | Dry weight of roots regenerated. | 1 gm. dry weight of leaves produced. | |
|---|---|---|---|---|---|
| | | | | Shoots. | Roots. |
| | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* |
| Set 1, *a*..... | 1.751 | 0.409 | 0.095 | 234 | 54 |
| *b*..... | 4.384 | 0.872 | 0.248 | 199 | 57 |
| " 2..... | 6.060 | 1.216 | 0.349 | 201 | 58 |

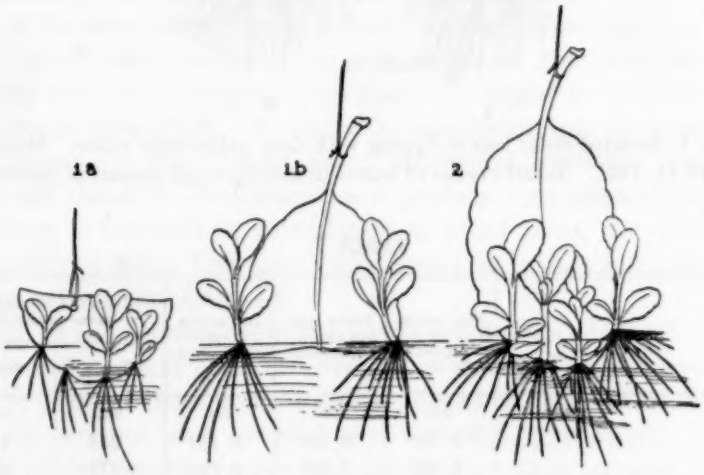



FIG. 2. One leaf (2) left intact, the sister leaf cut into two pieces, a small apical one (1*a*), and a larger basal one (1*b*). Shoot and root production are in proportion to the mass of the pieces. March 26 to April 17, 1923.

The experiment shows that the mass (in dry weight) of shoots and roots regenerated by isolated sister leaves of *Bryophyllum* varies under equal conditions approximately with the mass (in dry weight) of the leaves. On the assumption that the mass of material produced by assimilation in sister leaves, under the influence of light, varies with

the mass of the leaves, it is suggested that the quantity of regeneration is determined by the law of mass action.

That the root and shoot production in the small apical pieces (1*a*) is comparatively a little greater than in the large pieces is probably due to the fact that the sap has not so far to travel to reach the notches

TABLE III.

| | Dry weight of leaves. | Dry weight of shoots regenerated. | Dry weight of roots regenerated. | 1 gm. dry weight of leaves produced. | |
|---|---|---|---|---|---|
| | | | | Shoots. | Roots. |
| | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* |
| Set $a_1$ (in light)..... | 3.335 | 0.838 | 0.288 | 251 | 86 |
| " $a$ (" dark)..... | 2.445 | 0.102 | 0.008 | 42 | 3 |

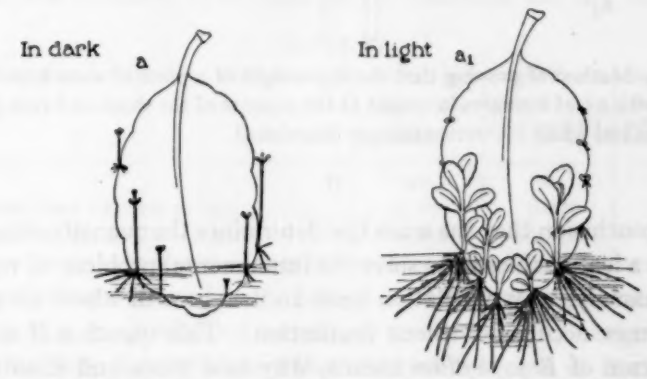



FIG. 3. Influence of light on mass of shoot and root production from detached leaves, one leaf ($a$) kept in dark, sister leaf ($a_1$) kept in light. The mass of shoot and root production in the dark is only a small fraction of that in light. March 13 to April 4.

where growth occurs in the small piece (1*a*) as in the larger pieces of leaf (2 and 1*b*).

*Experiment 3.*—To complete the proof, it is necessary to show that this regeneration depends partly or chiefly on the assimilating effect of the light. Twelve pairs of sister leaves were used, all of which were suspended so that their apices dipped into water as in Fig. 1. One leaf ($a_1$) of each pair was exposed to scattered daylight, while the twelve sister leaves ($a$) were kept in the dark but at the same

temperature. The experiment lasted from March 13 to April 4. The total mass of shoots and roots produced in the dark was less than 14 per cent of that produced in the light during the same time (Fig. 3 and Table III).

We may, therefore, draw the conclusion that the quantity of regeneration of a detached leaf of *Bryophyllum* (measured in dry weight of regenerated organs) is determined chiefly by the mass of material produced by assimilation.

Not all the material assimilated in a leaf is, as a rule, used for regeneration. Part of the material is used for the increase of the mass of the leaf. The data proving this fact may be omitted in this paper.

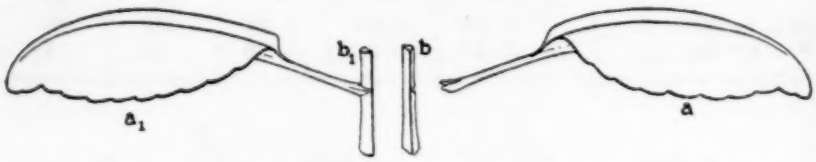



FIG. 4. Method of proving that the dry weight of a piece of stem kept in connection with a leaf increases in weight at the expense of the shoot and root production of the leaf which is correspondingly diminished.

II.

The conclusion that the mass law determines the quantity of regeneration in a leaf enables us to solve the fundamental problem of regeneration; namely, why mutilation leads to new growth where no growth would have occurred without mutilation. This question if adapted to the case of *Bryophyllum* means, why new roots and shoots arise from the notches of a leaf as soon as this leaf is detached from the plant, and why no such growth arises from the leaf as long as it is connected with a healthy plant. The answer is that as long as the leaf is part of a healthy plant, the greater part of the material formed in the leaf by assimilation goes into the stem and is used here for the normal growth of the plant. When the leaf is detached, this material becomes available for new growth (regeneration) of roots and shoots in the notches of the leaf. The method of proving this fact has already been described.[2] Pieces of a stem of *Bryophyllum* containing one node with two leaves each were cut out from a plant. Each piece of stem was split longitudinally through the middle (Fig. 4), to make the

two pieces of half stem ($b$ and $b_1$) as much alike as possible. In order to minimize the error in cutting, a larger number of pieces of stem were used for one experiment. One half stem (Fig. 4, $b$), was removed at once from one of each pair of sister leaves, and the dry weight of these half stems ($b$) was determined immediately. The two sets of sister leaves (one with a half stem attached, the other without a half stem) were suspended for several weeks in moist air, their apices dipping into water. It was found that the leaves ($a_1$) with a half stem ($b_1$) attached formed a smaller mass of shoots and roots than the leaves without such a piece of stem. At the end of the experiment, the dry weights of the leaves, shoots, roots, and of the half stems which had been left in connection with the leaves were determined. It was found that the dry weight of the half stems ($b_1$) left in connection with the leaves ($a_1$) had increased and that this increase in weight was sufficient to account for the excess in the dry weight of

TABLE IV.

| | Dry weight of leaves. | Dry weight of shoots regenerated by leaves. | Dry weight of roots regenerated by leaves. |
|---|---|---|---|
| | gm. | gm. | gm. |
| Set I (with stem attached to leaf)..... | 2.991 | 0.427 | 0.132 |
| " II (without stem)..... | 3.116 | 0.939 | 0.273 |

roots and shoots formed in the leaves ($a$) without pieces of stem. In other words, the inhibitory action of the stem on the regeneration in the leaf was due to the fact that the leaf sent part of its material into the stem, which otherwise would have been available for regeneration in the leaf.

In such experiments the axillary bud of the half stem left in connection with the leaf grows out and it is natural to infer that this growth withdraws material from the stem. It can be shown, however, that the stem inhibits the regenerative growth in the leaf also if this axillary bud is removed at the beginning of the experiment; and that, in this case also, the inhibitory influence of the piece of half stem on regeneration is due to the fact that the leaf sends part of the material produced by assimilation into the stem. In the stem it is used chiefly for callus formation at the basal end and for increase in thickness as well

as for longitudinal growth of the piece of stem (Fig. 5, I). The data in Table IV show the inhibiting effect of the piece of half stem attached to the leaf on the quantity of regeneration in the leaf.

*Experiment 4.*—Nineteen pairs of sister leaves were used. The pieces of half stem attached to one of each pair of sister leaves were about 25 mm. long.

The total dry weight of regenerated roots and shoots was 559 mg. in Set I and 1,212 mg. in Set II.

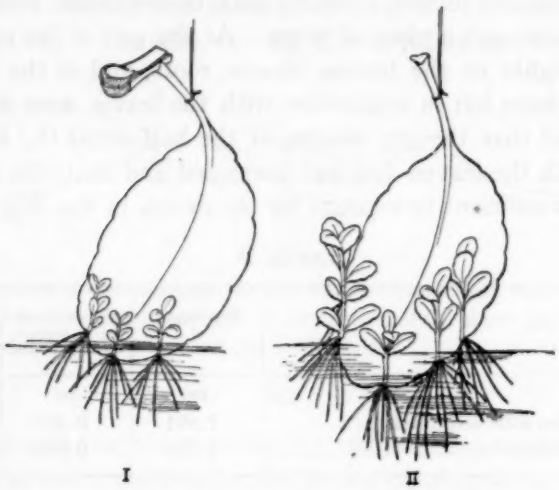



FIG. 5. Leaf I with a small piece of stem attached produces a smaller mass of shoots and roots than the sister leaf II without stem. The piece of stem increases in mass, especially through callus formation, and this accounts for the inhibiting action of the stem on shoot and root formation in leaf. Axillary bud of stem removed. April 6 to April 26.

Hence the presence of the small piece of stem in Set I (Fig. 5) diminished the quantity of regeneration in the nineteen leaves of this set by 653 mg. (The correction for the slight difference in the mass of the two sets of leaves reduces this value to about 630 mg.)

The dry weight of the nineteen half stems determined at the beginning of the experiment was 0.747 gm. The dry weight of the nineteen half stems (which were left in connection with the leaves) determined at the end of the experiment was 1.213 gm. Hence the stems connected with the leaves gained 466 mg. Since this gain

must have occurred through material furnished by the leaf, the inhibitory influence of the stem on the regeneration in the leaf is within the limits of accuracy of the experiments accounted for by the flow of material from the leaf into the stem.

When the axillary bud of the half piece of stem is not cut out, but allowed to grow, the flow of material from the leaf into the stem is

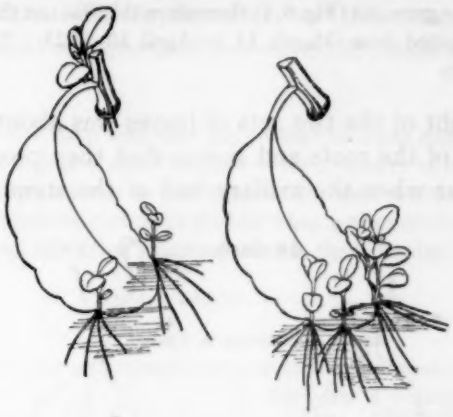



FIG. 6. The inhibitory action of a piece of stem on root and shoot formation in leaf is greater when the axillary shoot can grow out as in I than in sister leaf II where the axillary bud of the stem was removed. March 13 to April 9.

TABLE V.

| | Dry weight of leaves. | Dry weight of shoots regenerated by leaves. | Dry weight of roots regenerated by leaves. |
|---|---|---|---|
| | *gm.* | *gm.* | *gm.* |
| Set I (with axillary shoot)..... | 1.745 | 0.056 | 0.027 |
| " II (without axillary bud)..... | 1.754 | 0.267 | 0.068 |

much more considerable; and, accordingly, the inhibitory influence of the stem on regeneration in the leaf is still more considerable as is shown in the following experiment.

*Experiment 5.*—Pieces of stem about 25 mm. long with one node each possessing two leaves were cut out and the pieces of stem were split longitudinally as nearly in the middle of the stem as possible (Fig. 6). In this case, none of the pieces of stem were removed; but in one set of leaves the axillary buds of the stem were

removed (Fig. 6, II), while in the other set of leaves the axillary bud was not removed (Fig 6, I). The leaves were suspended in an aquarium, dipping with their apices into water. The axillary shoots grew out in eleven of the stems and these and their sister leaves with stems, the buds of which were cut out, were selected for a measurement of the influence of the growth of the axillary bud on the regeneration in the leaf (Fig. 6). It was found that the inhibitory effect of the half stems on the regeneration in the leaf was greater when the axillary bud of the stem was allowed to grow out (Fig. 6, I) than when this was not the case (Fig. 6, II). The experiment lasted from March 13 to April 10, 1923. Table V gives the quantitative results.

The dry weight of the two sets of leaves was about the same, but the dry weight of the roots and shoots that they produced was considerably smaller when the axillary bud of the stem was allowed to

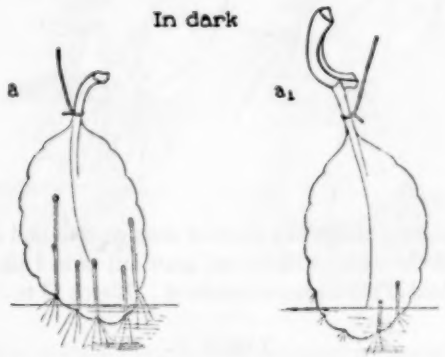



FIG. 7. The inhibitory effect of the stem occurs also in the dark, leaf *a* producing a greater mass of shoots and roots than its sister leaf $a_1$, which has a piece of stem attached. April 18 to May 9.

grow than when this was not the case. The leaves of Set I, Fig. 6, the stems of which formed axillary shoots, produced in all only 83 mg. dry weight of roots and shoots, while the leaves of Set II, Fig. 6, the stems of which formed no axillary shoots, produced in all 335 mg. of roots and shoots; *i.e.*, four times as much. This difference is accounted for by the weight of the eleven axillary shoots formed in the stems of Set I, which was 0.454 gm.

In these experiments the piece of stem connected with a leaf was very small, only about 25 mm. When the piece of stem is large, it can absorb more material and as a consequence has a greater inhibiting

power on shoot and root production in a leaf. Figures supporting this statement have already been given and the later drawings in this paper, *e.g.* Figs. 8 to 12, show that leaves when connected with large pieces of stems form no roots and shoots, at least not in the restricted time of these experiments.

In the experiments referred to in this paper the apex of the leaf dipped into water. This is, however, not a necessary prerequisite; what has been said remains true also when the leaves are suspended in moist air.

*Experiment 6.*—We have seen that the amount of shoot and root formation of a leaf in the dark is only a small fraction of the quantity of regeneration in the light. It was next of interest to find out whether there occurs in the dark too a diminution of the shoot formation in the leaf when a piece of stem is attached to it and whether such an inhibition is accompanied in the dark also by a corresponding

TABLE VI.

*Dark Experiment.*

| | Dry weight of leaves. | Dry weight of shoots regenerated by leaves. | Dry weight of roots regenerated by leaves. |
|---|---|---|---|
| | gm. | gm. | gm. |
| Set $a$ (nine leaves without stems)..... | 1.035 | 0.070 | 0.007 |
| " $a_1$ ( " sister leaves with pieces of half stems attached)..... | 1.027 | 0.006 | 0.004 |

increase in the dry weight of a stem. It was found that the stems actually diminish or repress the already small amount of shoot formation of a leaf in the dark and that the dry weight of the stems increases to about the same amount in weight as the shoot formation in the leaf is diminished. Fig. 7 indicates the difference in the appearance of the leaves with and without stems attached when kept in the dark. The experiment lasted 20 days. The leaf $a$, without stem, produced more shoots than its sister leaf $a_1$ with a half piece of stem attached. The stems gained correspondingly in weight. The exact figures of the dry weight measurements of the experiment are given in Table VI.

The nine leaves ($a$) without stems, formed in all 77 mg. of shoots and roots, while the sister leaves ($a_1$) with a piece of half stem attached, formed in all 10 mg. of shoots and roots, a difference of 67 mg. The half stems had at the beginning a dry weight of 0.454 gm., while the half stems of $a_1$ had at the end of the experiment a dry weight of

0.505 gm., an increase of 51 mg. Hence the half stems in Set $a_1$ gained in the dark about enough in dry weight to account for the inhibitory effect of the stem of the leaf $a_1$ on regeneration in the leaf. In this experiment the stems formed no axillary leaf. In a repetition of the same experiment in the dark, in which seventeen pairs of leaves with half stems were used, seven of these leaves, in which the stems remained attached, formed axillary buds. As a consequence more material flowed from the leaves into the stems and the inhibition of shoot and root formation in the leaves with half stems attached became even more complete. Table VII gives the results. The experiment lasted 22 days.

The leaves without half stems produced in all 175 mg. more dry weight of shoots and roots than the leaves with half stems attached in which the inhibition of root and shoot formation was almost

TABLE VII.

*Dark Experiment.*

| | Dry weight of leaves. | Dry weight of shoots regenerated by leaves. | Dry weight of roots regenerated by leaves. |
|---|---|---|---|
| | gm. | gm. | gm. |
| Set *a* (seventeen leaves without stems)..... | 1.740 | 0.161 | 0.019 |
| " *b* ( " sister leaves with pieces of half stems attached)..... | 1.732 | 0.005 | 0 |

complete. The dry weight of seventeen half stems at the beginning was 0.571 gm., at the end 0.879 gm. This latter figure included the weight of seven axillary shoots. Hence the dry weight of the stems increased by 308 mg., more than enough to account for the inhibitory action of the stems on shoot and root production in the leaves with half stems attached.

In these experiments the material sent by the leaf into the stem in the absence of light had been formed previously by the light. In this respect the regeneration of plants in the dark resembles the regeneration in starving animals, where the regeneration depends also on the hydrolysis of stored material.

We now understand why the leaf of *Bryophyllum calycinum*, when it is detached from the plant, forms shoots and roots from its notches,

while this regeneration is inhibited when the leaf forms part of a normal plant. The leaf connected with a normal plant can be dipped into water without forming roots or shoots in its notches. All the material available for shoot and root formation in the leaf is sent into the stem. During a recent visit in Bermuda, I have had a chance to examine thousands of plants of *Bryophyllum calycinum* without finding a single case where a leaf connected with a plant had formed roots or shoots. The same has been true in my greenhouse, and only recently have I had a chance to observe about six plants the older leaves of which formed some tiny shoots. The plants where this occurred were old and in two boxes containing no other plants; so that the suspicion is justified that they had suffered some common injury or disease. When a stem contains many leaves, and when the growth of the stem is stopped or when the sap flow has suffered, it is probable that shoots and roots may originate on leaves still connected with the stem. All that is needed for such growth is that the flow of material from the leaf into the stem should be partially or completely prevented.

The fact that in such cases regeneration can occur in leaves connected with a stem and hence without injury eliminates the idea that "wound hormones" or "wound stimuli" are the cause of shoot and root formation in the notches of a detached leaf of *Bryophyllum*.

This then solves the first part of the problem of regeneration, namely, the correlation of the new growth with the mutilation, and the solution is this, that as a consequence of the mutilation the sap and the solutes it contains collect in places where they could not have collected without the mutilation. The quantitative method of experimentation made it possible to prove this correlation.

This correlation holds also for regeneration in the stem. A defoliated piece of stem cut out from a healthy plant of *Bryophyllum calycinum* forms shoots from the most apical buds and roots at the base. In a preceding paper[3] it had been shown that the dry weight of roots and shoots formed in such a defoliated stem increases approximately with the mass of the stem, when the latter is exposed to light. Since the stem contains chlorophyll, this quantitative cor-

[3] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 447.

relation between mass of stem and mass of regenerated roots and shoots suggests that the mass law controls regeneration in this case also. This is supported by the fact that in the dark regeneration of roots and shoots by the stem is as considerably diminished as it is in the case of the regeneration of the leaf.

III.

In the case of a defoliated stem, there enters the phenomenon of polarity inasmuch as shoots are only formed at the apex and roots only at the base of the piece of stem. In this case, regeneration may be said to lead to a semblance of restoration of the original form of the mutilated organism, and this leads us to the second problem of regeneration; namely, to explain the polar character of the regeneration.

Since the mass of roots as well as of shoots regenerated in a piece of stem increases in direct proportion with the mass of the stem, it is obvious that the whole mass of material available for regeneration in the stem must be active in both shoot and root formation. If, nevertheless, shoots are only formed at the apex, one or more additional factors must be responsible for the fact that shoots are not also formed at the base. Two possibilities present themselves: first, the old assumption that there is a chemical difference in the nature of the substances fit for root and shoot formation, the material fit for shoot formation moving only in the ascending current, while the descending current carries root-forming material. A second possibility is that the material of each, ascending as well as descending, sap can give rise to both types of organs, but that the anlagen for root and shoot formation are contained in different histological layers of the stem; the cell layers where roots are formed being normally reached only by the descending sap and the layers where the shoots are formed being normally reached only by the ascending sap.

Specific root- and shoot-forming substances are not yet definitely known to exist, and our knowledge concerning the difference in the location of the vessels for the descending and ascending sap is not sufficiently definite to decide between the two possibilities. It is possible, however, to prove experimentally that the sap sent out by a leaf in the descending stream can accelerate also the rate of growth of

shoots in the more basal parts of the stem, provided such basal shoots are given a chance to grow.

*Experiment 7.*—In Fig. 8, *a* is a piece of stem without leaf, *b* a piece of stem with a reduced piece of leaf attached to the apex, and *c* a stem with a whole leaf attached to the apex. The right side of the upper part of the stem opposite the leaf is cut off. The stems of *b* and *c* have formed roots at the base, but only on that side of the stem where the leaf is, showing that the material for root formation was carried in the descending current from the leaf. Moreover, the mass of roots is greater

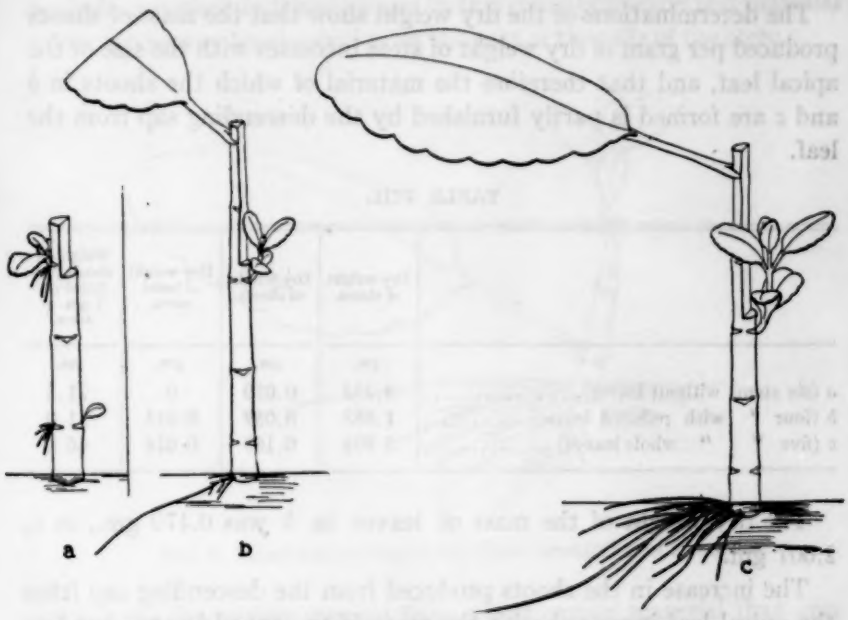



FIG. 8. Proof that the descending sap from a leaf can also under proper conditions increase shoot production. The shoot production in *c* with a whole apical leaf attached is greater than in *b* with only a piece of leaf attached. Without leaf, in *a*, the shoot production is a minimum. Roots at the base form in *b* and *c* first on that side of the stem where the leaf is. December 7 to January 5.

in *c* than in *b* corresponding to the difference in the mass of the leaf. Later on, however, roots may form in the whole circumference of the base of the stem. Stem *a*, which had no leaf, has not formed any roots at the base, but only the transitory air roots in nodes, these air roots disappearing when the permanent roots at the base are formed (as had been shown in a previous publication).[4]

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 687.

All three stems have formed shoots, but the shoot is greatest in *c* where the mass of the leaf is greatest, is smaller in *b* where the leaf is reduced in size, and smallest in *a* where there is no apical leaf. In other words, it seems as if the shoots in *b* and *c* had at least been partly produced from material sent in the descending current from the apical leaf. This inference was supported by quantitative determinations of the dry weight (Table VIII).

All these experiments were carried on simultaneously and lasted from December 7, 1922 to January 5, 1923.

The determinations of the dry weight show that the mass of shoots produced per gram of dry weight of stem increases with the size of the apical leaf, and that therefore the material of which the shoots in *b* and *c* are formed is partly furnished by the descending sap from the leaf.

TABLE VIII.

| | Dry weight of stems. | Dry weight of shoots. | Dry weight of basal roots. | Weight of shoots produced per 1 gm. of stem. |
|---|---|---|---|---|
| | *gm.* | *gm.* | *gm.* | *gm.* |
| *a* (six stems without leaves) | 3.252 | 0.070 | 0 | 21.5 |
| *b* (four " with reduced leaves) | 1.883 | 0.059 | 0.014 | 31.0 |
| *c* (five " " whole leaves) | 3.934 | 0.180 | 0.074 | 46.0 |

The dry weight of the mass of leaves in *b* was 0.470 gm., in *c*, 2.607 gm.

The increase in the shoots produced from the descending sap from the apical leaf increased with the mass of the apical leaves, but less rapidly. The mass of the basal roots increased, however, almost in direct proportion with the mass of the leaf.

Part of the material sent out by the leaf in the descending current is utilized for the growth in length and thickness of the more peripheral tissues of the stem from which the roots originate, leaving only a fraction of the material of the descending current free to be utilized for the growth of the shoots in *b* and *c* of Fig. 8.

This is supported by the following fact also observed in Experiment 7 that when a leaf is left at the base of a small piece of stem, as in Fig. 9, the stem produces per gram dry weight more apical shoots

than were produced in *b* or *c* of Fig. 8. 1.869 gm. dry weight of stems, each with a basal leaf, produced 0.348 gm. dry weight of apical shoots, or 183 mg. of apical shoots per gm. of stem; in the same time and the same conditions the stem *c*, Fig. 8, produced only 46 mg. dry weight of shoots per gm. of stem when the leaf was at the apex. The dry weight of the basal leaves in Fig. 9 was only slightly more than in *c;* namely, 2.899 gm. instead of 2.607 gm. When the leaf is at the base, the material carried from the leaf to the apical shoot must move in the ascending current to the stem and in this case apparently less material is lost on the way by absorption on the part of the cells of the stem.

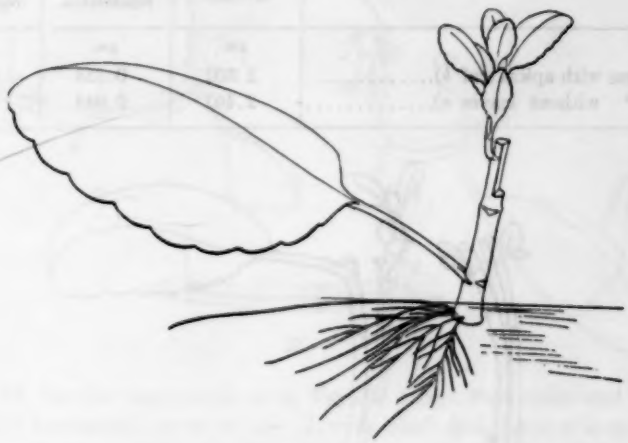


FIG. 9. Basal leaf promotes also shoot production at apex.

That the descending current from a leaf carries material that can be utilized for shoot formation can also be demonstrated in the following form of experiment.

*Experiment 8.*—In Fig. 10 the two stems *a* and *b* are split longitudinally from the apex down to near the base which dips into water. Stem *a* has no leaf, while stem *b* has an apical leaf to the right. The experiment lasted from January 12 to February 8, 1922. Stem *a*, without a leaf, formed no roots at the base in water, but two tiny shoots at the apex. Stem *b* formed roots at the base on that side only where the leaf is, and a shoot at the apical end of the stem on the opposite side of the leaf. This shoot exceeded in mass the two tiny shoots formed in *a*. The determinations of the dry weight confirmed this (Table IX).

The difference in the mass of shoots produced by *a* and *b* of Fig. 10 can only be ascribed to the material which was carried down from the leaf in the descending current to the base of the stem, rising afterwards on the other side of the stem. The dry weight of the six apical leaves of Set I was 3.388 gm.

In experiments of this kind, the mass of the apical leaf must be large in comparison with the mass of the stem, otherwise too much

TABLE IX.

| | Dry weight of stems. | Dry weight of shoots regenerated. | Dry weight of roots regenerated. |
|---|---|---|---|
| | *gm.* | *gm.* | *gm.* |
| I (six stems with apical leaf *b*)..... | 2.802 | 0.358 | 0.121 |
| II (five " without leaves *a*)..... | 2.462 | 0.044 | 0.002 |

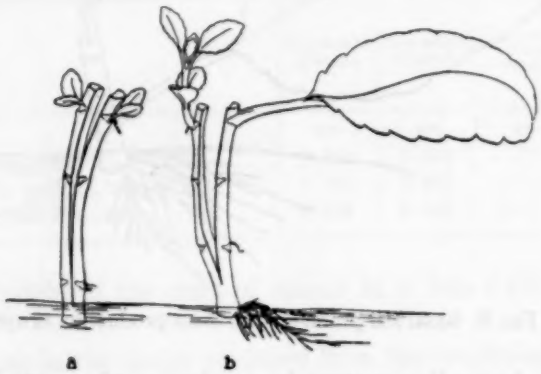



FIG. 10. Stems split longitudinally from apex almost to the base. Stem *b* with one apical leaf forms a shoot with greater mass than the stem *a* without leaf. Root formation in *b* first on side of leaf. January 12 to February 8, 1922.

of the material from the leaf is consumed in the descending current by the cortical tissues of the stem. This was ascertained by special experiments which may here be omitted.

*Experiment 9.*—Three sets of four small stems each were split longitudinally and suspended horizontally (Fig. 11). Set I had a large leaf at the apex, Set II a reduced leaf, and Set III had no leaf. All produced shoots at the upper apical node, but, as Fig. 11 shows, the size of the shoots increased with the size of the

apical leaf. In this case, the sap from the leaf had to travel in the descending current to the base of the stem and then on the other side of the split back to the apex. There can be no doubt that the descending current from the apical leaf favored shoot formation. In Set I, 1 gm. dry weight of stem produced 57 mg. dry weight of shoot, in Set II, 37 mg., and in Set III, which had no leaf, only 12 mg.

An interesting modification of the experiment is that represented in Fig. 12, *a*. A piece of stem with one leaf left at the apex was split lengthwise at some distance beneath the leaf, until near the base, which dipped into water. The apical bud opposite the leaf was

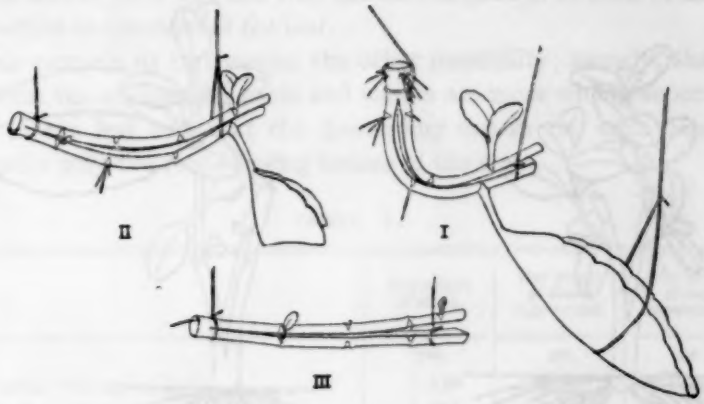



FIG. 11. Similar experiments as in Fig. 10, except that stems and leaves are suspended horizontally in moist air. I, with whole leaf, forms a larger shoot at apex than II with a leaf reduced in size. III, without leaf, produces only tiny shoots. Notice also that geotropic curvature of stem increases with mass of leaf. April 3 to April 21, 1923.

removed as indicated diagrammatically in the figure. In this case the descending sap had to flow down the stem on the side opposite the leaf and on this side the first roots developed at the base, spreading finally, however, all around the base. No shoot developed on that side (Fig. 12, *a*). A shoot developed, however, on the same side where the leaf is, at the apex of the split part of the stem (Fig. 12, *a*). Now the question arose, whether or not the descending sap of the leaf contributed to the growth of this shoot. This turned out to be true as the following quantitative experiment shows.

Five stems without leaves were split lengthwise down to near the base, as shown in Fig. 12, *b*, each split half producing a shoot at the apex. Six stems of almost the same mass but with one leaf left at the apex, as in Fig. 12, *a*, were split as indicated (Fig. 12, *a*). The latter stems produced one shoot at the apex of the split half of the stem as indicated in Fig. 12, *a*, and occasionally a second smaller shoot in the node below. Now the total dry weight of shoots produced by the six stems in *a* was 1.557 gm., while the total dry weight of shoots produced by the five stems in *b* without leaves was only 0.668 gm. The dry

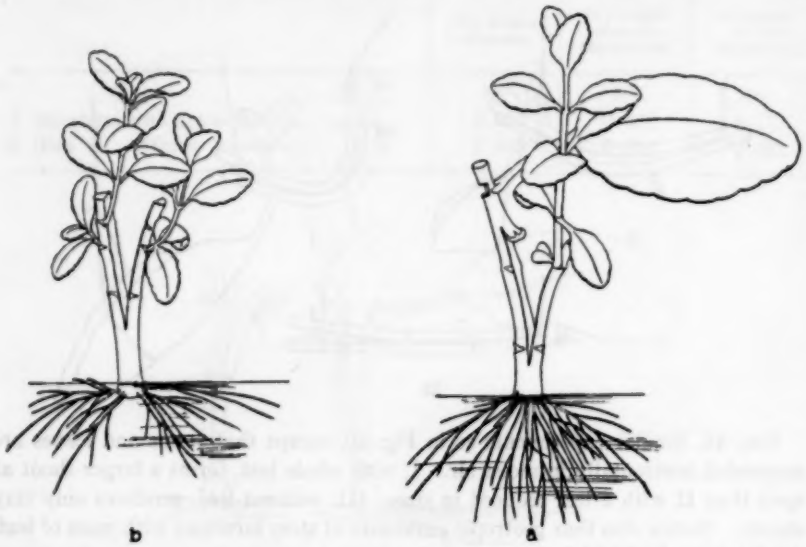



FIG. 12. Stem with leaf attached produces a greater mass of shoots than stem without leaf. January 9 to March 16, 1923.

weight of the six apical leaves was 2.063 gm., about the same as that of the stems. This leaves no doubt that the descending sap from the apical leaf contributed to the growth of the shoots below the leaf in *a*. In this case, the descending sap had to travel down the whole length of the stem on the side opposite the leaf (Fig. 12, *a*), and had to then ascend again to the apex of the split part of the stem. The exact figures are given in Table X. The mass of regenerated shoots is so great because the experiment lasted longer than usual; namely, over 2 months (January 9 to March 16).

These and other experiments leave no doubt that the sap sent out by a leaf in the descending current through the stem contains material fit for shoot production. According to Sachs the descending current should carry only root-forming substances and this makes it difficult to explain the second problem of regeneration—which, in this case, is the polar character of regeneration—on the assumption that the descending sap carries only specific root-forming substances. That the sap of the leaf is fit to give rise to both roots and shoots is also demonstrated by the fact that in each notch of an isolated leaf roots as well as shoots grow out and that the rate of growth of both occurs in proportion to the mass of the leaf.

This compels us to consider the other possibility; namely, that in the stem the anlagen for roots and shoots are more widely separated than in the leaf and that the descending sap in the stem reaches primarily only the root-forming tissues of the stem.

TABLE X.

| | Dry weight of stems. | Dry weight of shoots regenerated. | Dry weight of roots regenerated. |
|---|---|---|---|
| | *gm.* | *gm.* | *gm.* |
| *a* (six stems with apical leaf)..... | 2.139 | 1.557 | 0.306 |
| *b* (five " without leaves)..... | 1.987 | 0.668 | 0.040 |

## V.

## SUMMARY AND CONCLUSIONS.

1. The writer's older experiment, proving that equal masses of isolated sister leaves of *Bryophyllum* regenerate under equal conditions and in equal time equal masses (in dry weight) of shoots and roots, is confirmed. It is shown that in the dark this regeneration is reduced to a small fraction of that observed in light.

2. The writer's former observation is confirmed, that when a piece of stem inhibits or diminishes the regeneration in a leaf, the dry weight of the stem increases by as much or more than the weight by which the regeneration in the leaf is diminished. It is shown that this is also true when the axillary bud in the stem is removed or when the regeneration occurs in the dark.

3. These facts show that the regeneration of an isolated leaf of *Bryophyllum* is determined by the mass of material available or formed in the leaf during the experiment and that such a growth does not occur in a leaf connected with a normal plant for the reason that in the latter case the material available or formed in the leaf flows into the stem where it is consumed for normal growth.

4. It is shown that the sap sent out by a leaf in the descending current of a stem is capable of increasing also the rate of growth of shoots in the basal parts of the leaf when the sap has an opportunity to reach the anlagen for such shoots.

5. The fact that a defoliated piece of stem forms normally no shoots in its basal part therefore demands an explanation of the polar character of regeneration which lays no or less emphasis on the chemical difference between ascending and descending sap than does Sachs' theory of specific root- or shoot-forming substances (though such substances may in reality exist), but which uses as a basis the general mass relation as expressed in the first three statements of this summary.

6. It is suggested that the polar character of the regeneration in a stem of *Bryophyllum* is primarily due to the fact that the descending sap reaches normally only the root-forming tissues at the base of the stem, while the ascending sap reaches normally only the shoot-forming anlagen at the apex of the stem.

7. This suggestion is supported by the fact that when the anlagen for shoots and roots are close together as they are in the notch of a leaf, the sap of the leaf causes the growth of both roots and shoots from the same notch and the influence of the sap of the leaf on this growth increases for both roots and shoots in proportion with the mass of the leaf.

# THEORY OF GEOTROPISM BASED ON MASS ACTION.

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In a previous publication the writer had shown that when a piece of stem of *Bryophyllum* is cut out from a plant and suspended horizontally in a moist atmosphere, the stem bends, the lower side becoming convex, the upper concave. He was able to prove that this geotropic curvature is a phenomenon of growth, the cortical tissues of the lower side of the stem growing more rapidly in length than the cortical tissues on the upper side of the stem or than the tissues of the wood. He was also able to show that the geotropic curvature is more rapid when the piece of stem possesses one or two apical leaves than when it is free from leaves.[1]

If growth is a function of mass action, then this geotropic curvature of the stem must have its cause in the fact that a greater mass of the material required for growth must collect on the lower than on the upper side of a stem suspended horizontally. This can happen if the liquid—the tissue fluid—containing this material collects in greater quantity on the lower than on the upper side of the stem, or, in other words, if this tissue fluid follows gravity. In order to test the idea that geotropic curvature is a function of chemical mass action, it is necessary to find out, first, whether the mass of material sent by a leaf into a stem increases with the mass of the leaf, and second, whether the geotropic curvature of a stem suspended horizontally increases also with the mass of the leaf.

First, a simple experiment proves that a piece of stem gains more in dry weight when it is attached to a large than when it is attached to a small piece of leaf. Fifteen pieces of stem, each about 25 mm. long, with one node at the apex and a pair of leaves in the node were split longitudinally, and as accurately as possible in the middle,

[1] Loeb, J., *Bot. Gaz.*, 1917, lxiii, 25.

so that the two pieces of stem had approximately equal masses. The leaf of one half stem was left intact, while that of the other was reduced in size (Fig. 1). Both leaves dipped into water. Fig. 1 shows the appearance of the stems after 12 days. The pieces of stem attached to a large leaf had formed a callus and were geotropically bent, while the others had undergone no or little geotropic curvature and showed no or only traces of callus formation. At the end of the experiment, which lasted from April 13 to April 28, 1923, the dry weight of the whole leaves was 2.573 gm. and that of the reduced

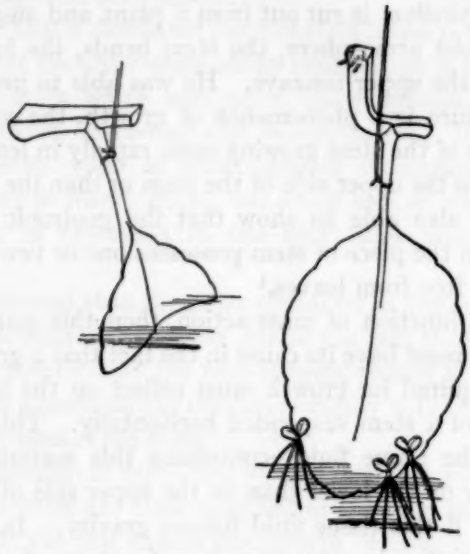

FIG. 1. Influence of mass of leaf on callus formation and geotropic curvature of stem. April 13 to April 25.

leaves 1.083 gm. The dry weight of the half stems attached to the whole leaves was 0.868 gm. while that of the half stems attached to the reduced leaves was only 0.765 gm. Hence the larger leaves sent 103 mg. more material (by dry weight) into the stems than did the smaller leaves. This mass of material which goes from the leaf into the stem is here used for callus formation and for that growth which gives rise to geotropic curvature when the stem is suspended horizontally. The next problem was to find out more accurately whether or not the curvature of a stem suspended horizontally increases with the mass of the apical leaf.

In such experiments it is necessary to remember that the geotropic curvature of a stem is the resultant of two opposing forces. One is the excess of longitudinal growth of cortical tissue on the lower side of the stem suspended horizontally over that of the rest of the stem; the opposing force is the rigidity of the upper layers of the stem, chiefly the wood. When the wood is too hard, the stem cannot bend. The influence of equal masses of an apical leaf on the rate of geotropic curvature of two stems can only be equal if the rigidity of the wood is identical in the two stems, a condition which cannot often be fulfilled. Quantitative work of this kind must therefore be statistical; but it is only intended here to prove the validity of the mass law for geotropism in a semiquantitative way. It is necessary to select for experimentation only the more apical parts of the stems of young plants where the wood is still soft, or not too rigid, otherwise little or no curvature is possible. The method of the experiments is illustrated in Fig. 2 of this paper. Stems of about equal flexibility were selected and defoliated with the exception of one leaf at the apex. With the aid of a string around the petiole of the leaf, the stem was suspended in a moist aquarium. In order to secure a horizontal position of the stem, the latter was put on a wire netting bent in the shape of a U on the bottom of which the stem was allowed to rest (Fig. 2). The meshes of the net were $\frac{1}{2}$ inch and the squares of the wire netting made it easy to follow and measure the slope of geotropic curvature from day to day. It was found that under proper conditions this curvature increases with the mass of the leaf as intimated in Fig. 2. Three stems (*a*, *b*, *c*) were selected, each having a leaf at the apex. The leaves in *b* and *c* were partly cut off so that the mass of the leaves in *a*, *b*, and *c* was roughly in the ratio of $1:\frac{1}{2}:\frac{1}{4}$. The drawing was made after 11 days. The figure shows that the degree of geotropic curvature in the three stems increased with the mass of the apical leaf, being a minimum in *c* and a maximum in *a*. When the stem contains no leaf, the curvature is a very slow process.

It seemed more accurate to modify the experiment by always comparing the rate of curvature of two halves of a stem split longitudinally as in Fig. 3, *a* and *b*. A stem with a pair of apical leaves was split as accurately as possible in the middle between the two leaves and each half was suspended as shown in the figure. The

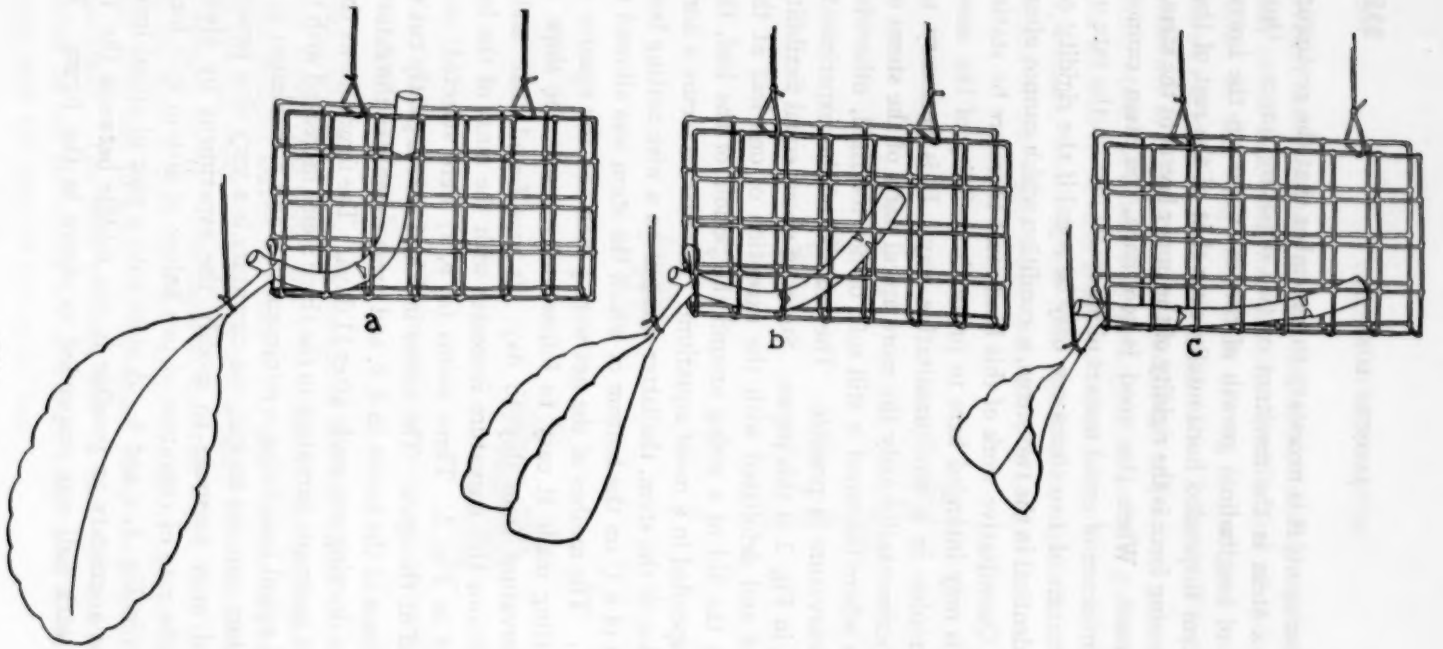


FIG. 2. Influence of mass of leaf on rate of curvature, *a* whole leaf, *b* half leaf, *c* about ¼ of a leaf. Curvature is greatest in *a*, least in *c*. April 27 to May 8.

leaf was on the lower side of the stem as in the preceding experiment, but while one leaf remained intact, part of the other was cut off, so that its mass was only about one-half of that of the whole leaf. Fig. 3 gives the curvature after 3 days, the curvature being greater where the mass of the leaf was greater. Fig. 4 gives the curvature of the two half stems a day later, and Fig. 5 on the 6th day.

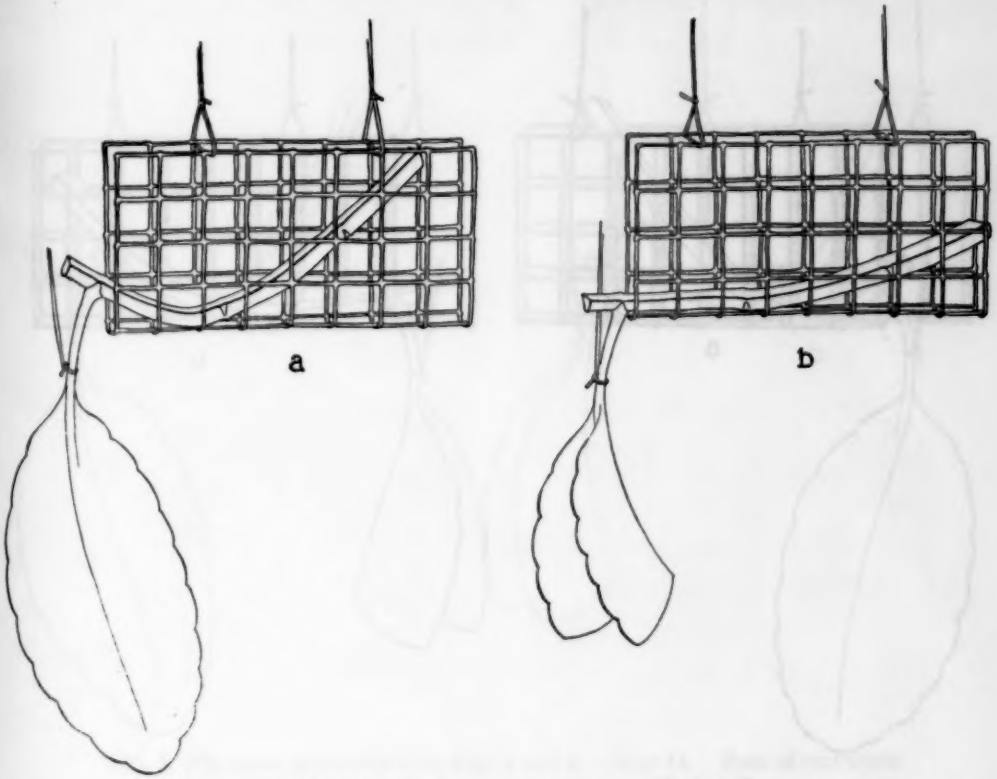


FIG. 3. Stem split lengthwise, one piece having a whole the other only half of a leaf. Curvature is greater in whole leaf. May 8 to May 11.

This experiment gives also an idea of the rapidity with which the curvature occurs at the temperature of the greenhouse (about 24°C.).

It may finally be of interest to show that the rate of geotropic curvature of these half stems with an apical leaf attached occurred approximately in proportion with the mass of the leaf. In Fig. 6

the curvature of three half stems each with a whole leaf was compared with the curvature of the three sister halves each with a reduced leaf. All the three stems with whole leaves had reached about the same curvature as stem *a*, and therefore this one alone is reproduced here. *b*, *c*, and *d* had bent approximately in proportion with the mass of the apical leaf attached.

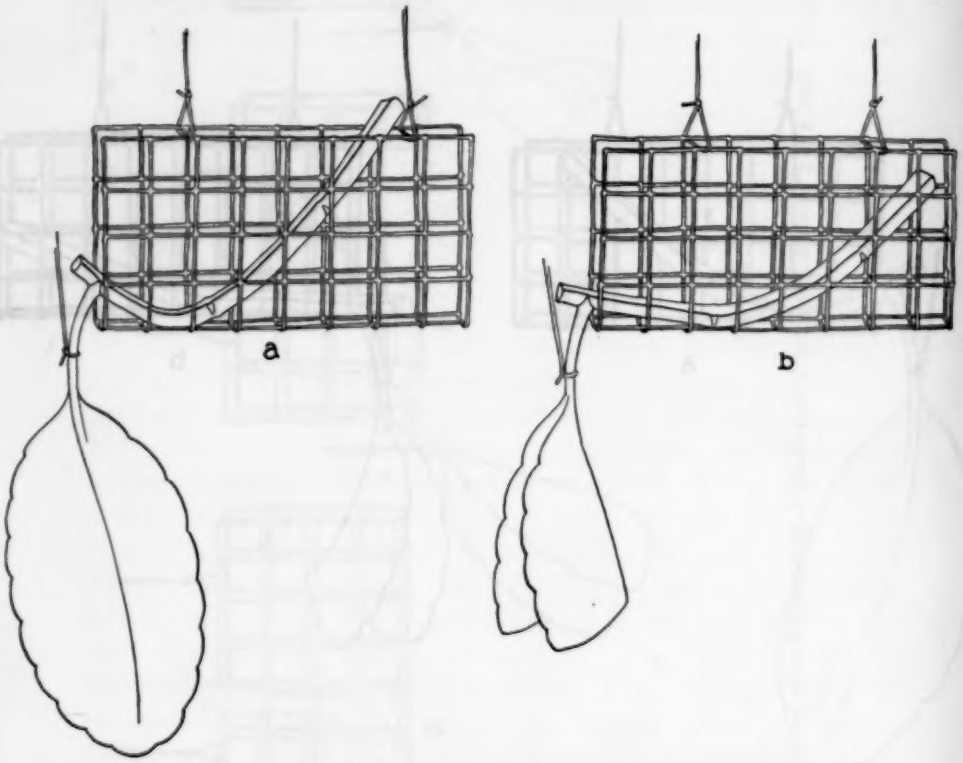


FIG. 4. The same specimens as in Fig. 3. May 12.

In Fig. 7 a stem was split longitudinally and both halves were suspended horizontally. One half had a leaf at the apex, while the other half had no leaf. The latter bent very slowly in comparison with the half which had an apical leaf attached, in accordance with the difference in the mass of material available for growth in the two half stems. It may be stated incidentally that the mass of shoots

and roots regenerated by the half stem with a leaf was also considerably larger than the mass of shoots and roots formed in the half stem without leaf. The experiment lasted from April 10 to May 23.

The experiments with split stems give on the whole less reliable results than those with whole stems. Not only the unavoidable

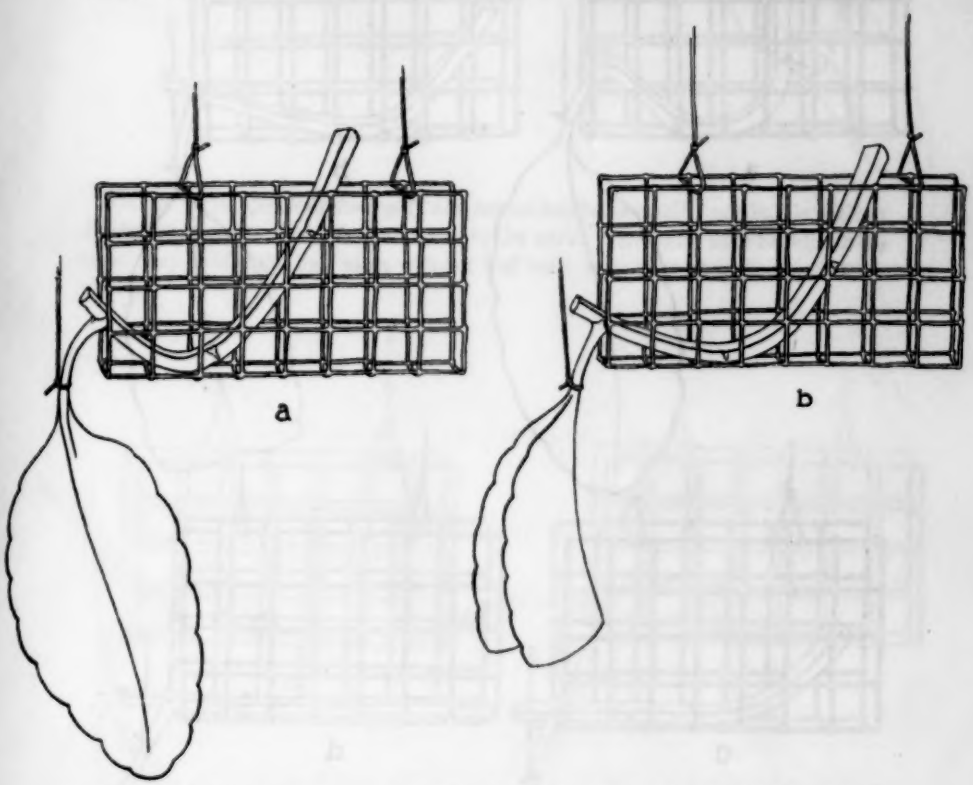


FIG. 5. The same specimens as in Figs. 3 and 4. May 14. Rate of curvature increases with mass of leaf.

errors in halving the stem, but possibly other variables vitiate the result, *e.g.* the unequal degree of drying of the upper side of the stem and the resulting inequalities in rigidity of the wood.

In all these experiments the leaf was at the apex of a piece of stem. When the leaf is at the base, no geotropic curvature ensues. This can be best demonstrated on stems split longitudinally which have

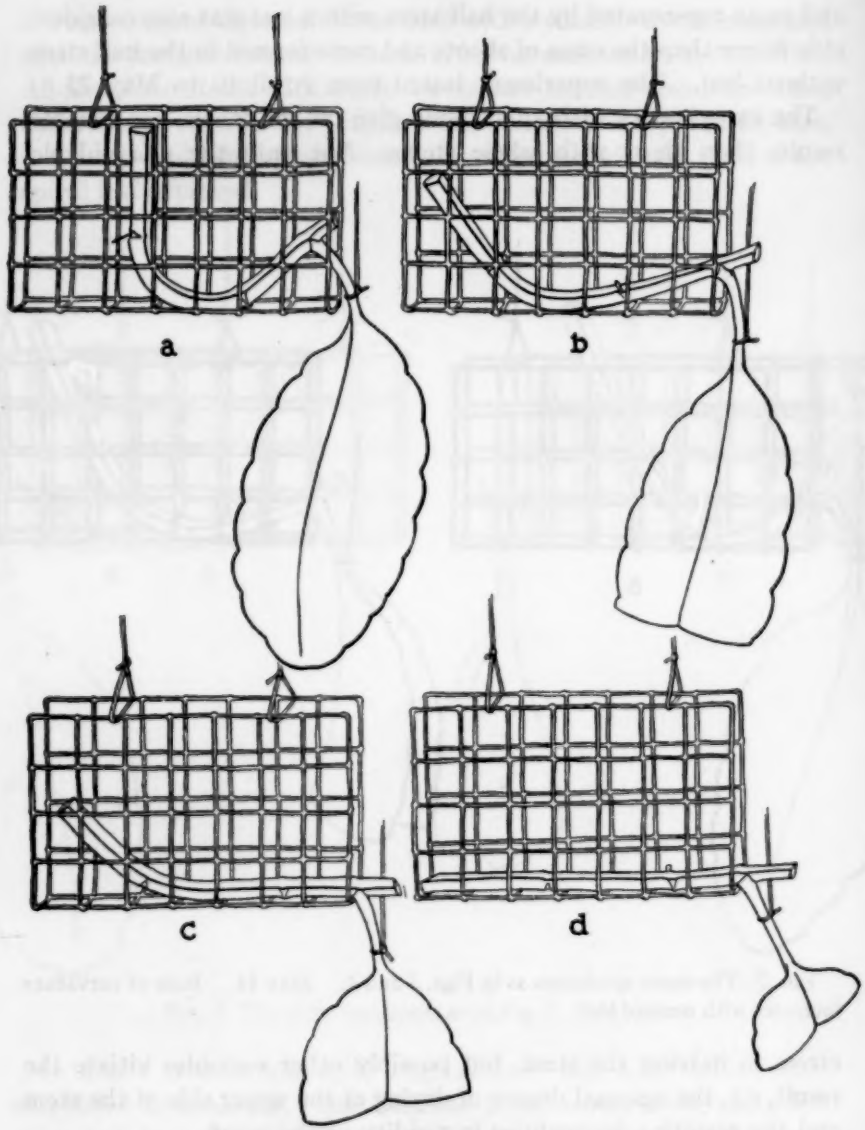


FIG. 6. *a*, piece of stem with whole leaf; *b*, *c*, and *d* with increasingly reduced leaf. The geotropic curvature increases with mass of leaf. May 8 to May 17.

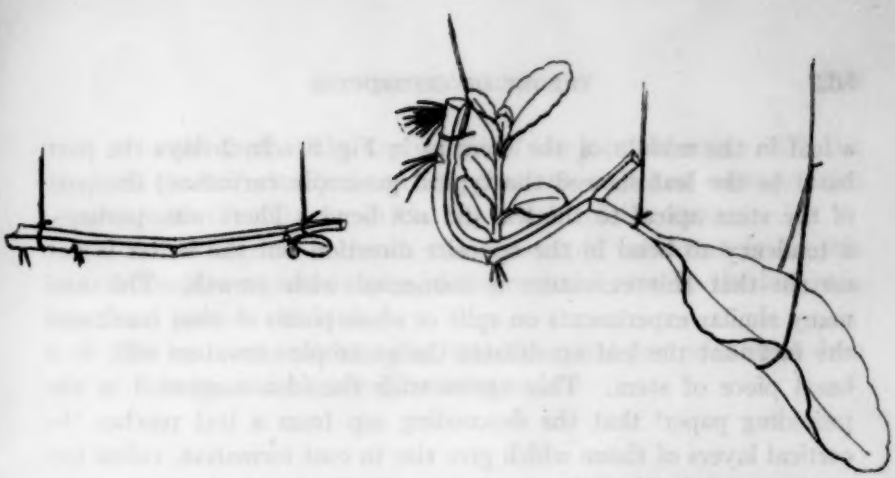

FIG. 7. Stem split longitudinally and suspended horizontally, one half is entirely defoliated, the other has a leaf attached to the apex. The latter bent rapidly in the usual way, while the half stem without leaf bent only very slowly. April 10 to May 23.

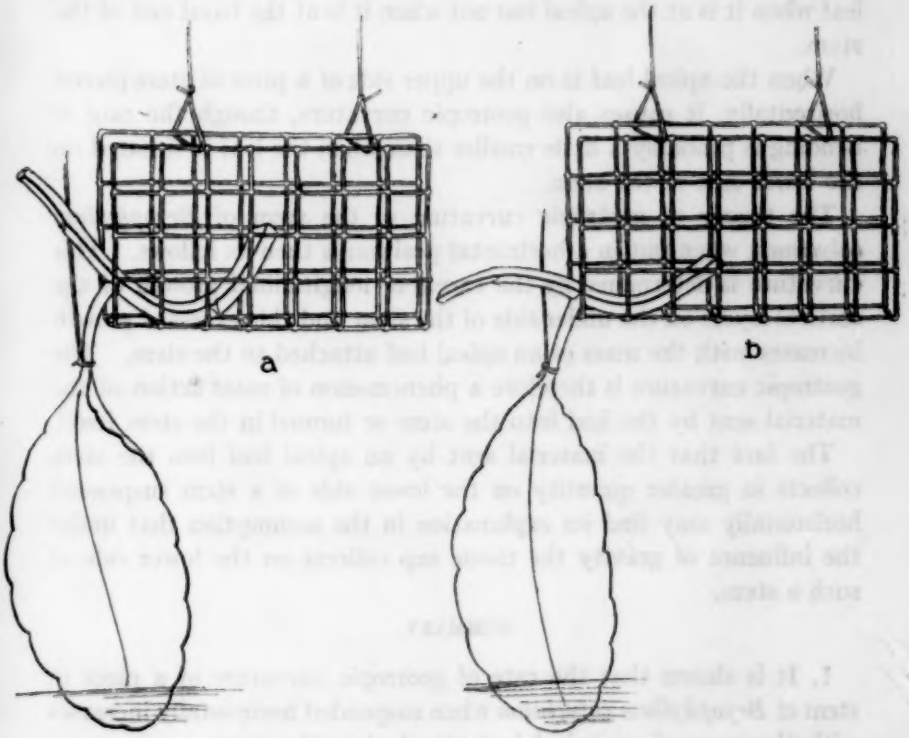


FIG. 8. Leaf in middle of stem. Geotropic curvature only in basal part of stem; the apical piece of stem shows no curvature or one in the opposite sense. May 14 to May 16.

a leaf in the middle of the stem as in Fig. 8. In 2 days the part basal to the leaf showed the typical geotropic curvature; the part of the stem apical to the leaf did not bend. There was, perhaps, a tendency to bend in the opposite direction but the writer is not certain that this curvature is connected with growth. This and many similar experiments on split or whole pieces of stem confirmed the fact that the leaf accelerates the geotropic curvature only in a basal piece of stem. This agrees with the idea suggested in the preceding paper[3] that the descending sap from a leaf reaches the cortical layers of tissue which give rise to root formation, callus formation, and incidentally to that longitudinal growth of the stem which causes geotropic curvature, while the ascending sap reaches only the most apical bud, causing it to grow into a shoot. Geotropic curvature of a horizontal stem is therefore accelerated only by a leaf when it is at the apical but not when it is at the basal end of the stem.

When the apical leaf is on the upper side of a piece of stem placed horizontally, it causes also geotropic curvature, though the rate of bending is probably a little smaller than when the leaf is situated on the lower side of the stem.

The theory of geotropic curvature of the stem of *Bryophyllum calycinum* when put in a horizontal position is then as follows. This curvature is determined by the excess of longitudinal growth of the cortical layers on the under side of the stem and this excess of growth increases with the mass of an apical leaf attached to the stem. The geotropic curvature is therefore a phenomenon of mass action of the material sent by the leaf into the stem or formed in the stem itself.

The fact that the material sent by an apical leaf into the stem collects in greater quantity on the lower side of a stem suspended horizontally may find its explanation in the assumption that under the influence of gravity the tissue sap collects on the lower side of such a stem.

## SUMMARY.

1. It is shown that the rate of geotropic curvature of a piece of stem of *Bryophyllum calycinum* when suspended horizontally increases with the mass of an apical leaf attached to the stem.

[3] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 831.

2. It is shown that the dry weight of the stem increases with the mass of the leaf attached and also that the degree of curvature increases with this increase in the dry weight of the stem.

3. The conclusion is drawn that geotropic curvature is in this case a function of mass action of the material sent by the leaf into the basal part of the stem.

4. The material sent by a leaf into the apical part of a stem does not lead to the same geotropic curvature.

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